THE ROLE OF PHENOTYPIC PLASTICITY AND LOCAL ADAPTATION IN ALPINE PLANTS FACING CLIMATE CHANGE

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Dekan

“A garden requires patient labor and attention. Plants do not merely grow to satisfy ambitions or to fulfill good intentions. They thrive because someone expanded effort on them.” - Liberty Hyde Bailey

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“If we knew what it was we were doing, it would not be called research, would it?” - Albert Einstein

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“[…] Find out the cause for this effect, /
Or rather say, the cause of this defect, /
For this effect defective comes by cause. “
– Polonius (Act2, Scene 2, line 104) Hamlet, Shakespeare

Contents

Chapter 1

General Introduction

General Introduction

Lectori salutem,

Before introducing the general aims, the main research questions, the experimental approach and the outline of this thesis, I would like to set its research frame, which revolves in the scientific field of plant population and evolutionary biology. For this purpose, I will first provide information about the environment of the Swiss Alps, its flora, and how it is threatened by climate change. I will then proceed to introduce terms such as evolution, natural selection, local adaptation, and phenotypic plasticity.

The Alpine flora and environment is threatened by climate change

Alpine biodiversity is particularly rich and the flora of the Alps comprises about 4'000 species (Aeschimann et al., 2004) and includes more than five hundred endemic species, i.e. unique to a particularly mountain region, where they have probably evolved. Plants had to adapt to the particular environmental conditions at high altitude (Körner, 2003). With increasing elevation, plant life is challenged in many ways, by extreme temperatures, a short vegetation period, snow, and by a rising number of weather-related extreme events (Körner, 2003). The alpine landscape is also characterized by great spatial and temporal environmental heterogeneity, creating a mosaic of micro-habitats (Scherrer and Körner, 2010, Scherrer and Körner, 2011). The environmental heterogeneity, along with the richness of endemics, highlights the strength of selective forces and evolutionary processes in the alpine landscape (Ozenda, 1988, Kadereit et al., 2008), making alpine flora clearly distinct of that of lowlands (Chapin and Koerner, 1995).

Climate change, well illustrated in Europe by increasing temperatures and changes in precipitation patterns, has been reported by the IPCC (Kovats et al., 2014) and it has been suggested that these effects are proportionally more pronounced at high elevation (Beniston et al., 1997). Indeed, in alpine regions the amplitude of temperature changes are greater then the observed global changes (Beniston et al., 1994). While a 0.7°C rise in air temperatures has been reported globally, a 2°C change in temperature has been recorded in the Alps (Auer et al., 2007). Additionally, summer droughts are predicted to become more frequent in many regions including mountain areas (Kovats et al., 2014), leaving mountain biota particularly vulnerable to climate change (Theurillat and Guisan, 2001, Körner, 2003).

In this context, it becomes increasingly important to investigate how the alpine flora will respond to environmental changes and evolve in a future climate.

A brief introduction to local adaptation and phenotypic plasticity

Evolution, the heritable change over time in the phenotype of an organism (Darwin, 1859) and natural selection, the process which selects for particular phenotypic variants in a population, have led to the adaptation of plants to their environment.

Within a species, populations may genetically differ through natural selection or random processes such as genetic drift. In widespread plants, the heterogeneity of habitat conditions over large spatial scales

may lead to changes in the selection pressures acting on functional plant traits and may thereby result in adaptive genetic variation in a way that maximizes fitness in different environments (Briggs and Walters, 1997). Indeed, widespread species show high levels of variation (Bradshaw, 2006), and frequently perform well in a wide range of environmental conditions (Joshi et al., 2001, Santamaria et al., 2003). On the one hand, adaptations to climatic variation or other conditions that differ at a larger spatial scale (coarse-grained environmental variation) should easily be maintained by natural selection, while genetic adaptations to environmental variability at a more local scale (fine-grained environmental variation) may be hindered by gene flow (Kawecki and Ebert, 2004). Since the pioneer studies of Turesson (1922) and Clausen et al. (1941), patterns of intraspecific variability were the focus of many studies, and specialization to particular environmental conditions has been frequently demonstrated (Van Tienderen, 1991, Dudley, 1996, Van Tienderen, 1997, Pluess and Stöcklin, 2005, Fischer et al., 2008).

As a result, it is usually assumed that plants are locally adapted. Local adaptation is characterized by adaptive differentiation among populations. Plants can be locally adapted either constitutively via genotypic differences or via phenotypic plasticity, which is the range of phenotypes a single genotype can express as a function of its environment (Bradshaw, 1965). Genotypic variability and phenotypic plasticity can be considered as complementary mechanisms adjusting plants to environmental heterogeneity (Van Tienderen, 1991, Van Tienderen, 1997).

A central goal in ecological genetics has been to determine to what extent different phenotypes in different environments result from local adaptation, phenotypic plasticity or a combination of both (Conner and Hartl, 2004, Ghalambor et al., 2007, Franks et al., 2014). However, intraspecific differentiation in alpine plants is also strongly affected by the repeated oscillations during glaciations (Scheepens and Stöcklin, 2011, Scheepens et al., 2015). Thus, to some extent, phenotypic differentiation in alpine plants may be ecologically relevant and adaptive, but to some degree it may result from random evolutionary processes (e.g. genetic drift). There are not many studies on alpine plants that have rigorously tested hypotheses concerning local adaptation, either for elevational effects (Galen and Stanton, 1991, Byars et al., 2007, Byars and Hoffmann, 2009, Hautier et al., 2009), differences in snow cover (Stanton and Galen, 1997), or adaptation to contrasting habitats (McGraw, 1987, Leinonen et al., 2009). Mostly, local adaptation in these studies was demonstrated across wide climatic or elevational gradients or to contrasting habitats, but populations were rarely transplanted across their original field sites. At the local scale genetic adaptation to environmental variability may be hampered by gene flow or source sink relations among nearby populations (Stanton and Galen, 1997, Kawecki and Ebert, 2004). Nevertheless, differentiation among alpine populations has also been demonstrated at the micro-scale, indicating the strength of small-scale heterogeneity as a selective force for local adaptation (Shimono et al., 2009). In other cases, adaptation to small-scale environmental heterogeneity was missing (Byars et al., 2009). Furthermore, local adaptation is also contingent on factors other than spatial scale, such as the plant mating systems and reproductive mode (i.e. vegetative vs. sexual reproduction) due to their effects o the degree of genetic differentiation of populations (Kawecki and

Ebert, 2004).

Clearly, the generality of local adaptation in alpine plants cannot be concluded based on the few studies available (Leimu and Fischer, 2008) and the extent and manner by which it is influenced by scale-dependent environmental heterogeneity of the alpine landscape is poorly known. Specifically, there is only little knowledge of how alpine plants are adapted to the pronounced environmental heterogeneity of alpine habitats. Such knowledge is however particularly important when trying to predict how plants will react to climate change.

Phenotypic plasticity in plant species has received growing attention in the past decades (Bradshaw, 1965, Schlichting, 1986, Sultan, 1987, Thompson, 1991). The concept of phenotypic plasticity in evolutionary biology is widely accepted and there is little doubt about the important role of plastic responses of plants in heterogeneous environments (Pigliucci 2005). The current interest in phenotypic plasticity results in part from an urgency to predict species responses to global change (Valladares et al., 2006, Nicotra et al., 2010). Phenotypic plasticity may play a crucial role in the short-term adjustment to novel conditions and can promote long-term adaptive evolution by buffering against rapid change (Price et al., 2003, Nicotra et al., 2010, Richter et al., 2012).

How much phenotypic plasticity is adaptive and favored by natural selection, how much do costs and genetic correlations act as a limitation for plasticity, and how much plasticity is only a passive response to environmental cues are intensively discussed research questions (Via and Lande, 1985, van Kleunen and Fischer, 2005, Bradshaw, 2006). Nevertheless, there are well documented examples of adaptive plasticity in plants, i.e. heterophylly in shallow water (Cook and Johnson, 1968), the variability of internode length in response to shading (Dudley, 1996), or the variability of leaf traits in response to temperature (Scheepens et al., 2010). Phenotypic plasticity is also likely to facilitate adaptive evolution in new or changing environments (Ghalambor et al., 2007). But generally, there is still little empirical knowledge on how much variability of functional traits in different environments is due to genotypic variability and how much it is a result of adaptive plastic adjustments. Furthermore, there is shortage of studies on phenotypic plasticity in the field.

It is however important to remember that this ability has a genetic basis in itself and is limited by costs and constrains (DeWitt et al., 1998, Pigliucci, 2001, Givnish, 2002, van Kleunen and Fischer, 2005, Valladares et al., 2007). Plant populations and species differ greatly in phenotypic plasticity, mainly because plasticity is advantageous under some conditions and disadvantageous or not advantageous under others (Alpert and Simms, 2002). Plasticity is hypothesized to be favored when an environmental factor varies on the same spatial scale as the plant response unit, when the plant can respond to an environmental factor faster than the level of the factor changes, and when environmental variation is highly but not completely *predictable* (Via and Lande, 1985, Alpert and Simms, 2002). While a small number of studies have examined the potential for phenotypic plasticity in plant populations with varying levels of environmental heterogeneity, the results do generally not align with these predictions (Heschel et al., 2004, Franks, 2011). Other authors have examined differences in plasticity between population from low and high elevations. While Vitasse et al. (2013)

found lower phonological plasticity in high elevation deciduous tree species, Frei et al. (2014) found no differences in plasticity between low and high elevation populations. These results show that the evolution of phenotypic plasticity in response to environmental heterogeneity and its associated costs and constrains are complex, and further work is needed to improve our understanding of these dynamics.

The aim of this thesis

The key elements addressed in this thesis are threefold. We aim at (1) examining if phenotypic plasticity allows alpine plants to buffer the effects of climate change; (2) comparing the degree of phenotypic plasticity between high and low elevation plants; (3) understanding the mechanism of local adaptation in alpine plants through genetic and phenotypic differentiation at different spatial scales. Combining all three elements, the central goal of this thesis is to provide a better understanding of the role of phenotypic plasticity and/or genetic differentiation possibly leading to local adaptation in alpine species, and in the context of climate change we aim at inferring on the adaptive potential of alpine species to future climate.

Main research questions

The central question of this thesis is whether genetic and phenotypic differences allow alpine species to buffer against climate change. In this context, two main sets of research questions structure this thesis. (1) Do alpine species exhibit plastic adjustments in key functional plant traits in response to changes in environmental conditions predicted to be altered by future climate change (i.e. warming and drought)? And does the capacity for phenotypic plasticity in alpine species differ from that of lowland species? (2) To what extent does adaptive genotypic differentiation and phenotypic plasticity influence local adaptation of alpine plants? In this second part, the central hypothesis is that in fine-grained environmental variation, where individuals experience highly heterogeneous conditions at a small spatial and/or temporal scale, natural selection should favor high phenotypic plasticity (Alpert and Simms, 2002), while in the case of coarse-grained environmental variation, where organisms experience a more stable environment over their life time, natural selection should have led to genetic adaptation among populations (Joshi et al., 2001).

We addressed the first set of questions in **Chapter 2** and **3** on a large number of high and low elevation perennial herbaceous species exposed to changes in temperatures and water availability. **Chapter 4** makes for a relevant transition between the two main question as we investigated the flowering phenology and biomass allocation patterns to reproductive structures in a single alpine species, namely *Anthyllis vulneraria*, and inferred on patterns of past diversifying selection. The second question is considered in **Chapter 5**, **6**, and **7**, and in associated technical articles related in **Chapter 8** and **9**. Here, we used four alpine species differing in life strategies, namely *Poa alpina*, *Geum reptans*, *Anthyllis vulneraria* and *Arabis alpina* to examine patterns of local adaptation to present conditions in the Swiss Alps.

Experimental approach

The two essential tools used to address the main questions of this thesis, were common

garden experiments and reciprocal transplantation experiments.

Common garden experiments, used to investigate the plastic responses of species to changes in environmental conditions, related in **Chapter 2, 3 and 4** are ideal for this purpose as they allow the transplantation of study species to sites with a prospective warmer or colder climate while keeping other regional-scale abiotic factors such as photoperiod and local weather conditions constant (Haggerty and Galloway, 2011, Scheepens and Stöcklin, 2013, Frei et al., 2014). Common garden experiments, where plants from different source populations are grown in a single environment, have been used in pioneer works (Turesson, 1922, Clausen et al., 1941) to investigate the genetically based phenotypic differentiation. This approach coupled with molecular analysis allows the comparison of quantitative trait differentiation and genetic differentiation at neutral marker loci (Q_{ST}-F_{ST} comparisons) to test for the role of past selection in shaping observed patterns of population differentiation (**Chapter 4**). Moreover, by including treatments in common garden experiments, particular hypotheses concerning the ability of genotypes to respond plastically to environmental variation can be tested (Scheepens et al., 2010, Frei et al., 2011). Here, we have used this method to examine the plastic adjustments in key plant functional traits, such as flowering phenology (**Chapter 2 and 4**), leaf traits, and biomass allocation (**Chapter 3**) in response to changes in temperature and soil water availability, and one experiment additionally infers on patterns of past selection (**Chapter 4**).

While common gardens are ideal to investigate past selection patterns and genetic differentiation in phenotypic plasticity among selected populations, they cannot prove whether any observed differentiation is due to adaptation to current environmental conditions. Reciprocal transplantation experiments, in which plants from different source populations are transplanted into original field sites, can be used to provide evidence for local adaptation. With reciprocal transplantation experiments one can rigorously test for local adaptation using the "home vs. away", "local vs. foreign" or "sympatric vs. allopatric" criterion (Kawecki and Ebert, 2004, Blanquart et al., 2013). Confounding effects have been suggested to induce biases in the first two criteria. Indeed, the "home vs. away" criterion compares a deme's fitness across habitats, which can be confounded by habitat quality, and the "local vs. foreign" criterion compares deme's fitness across habitats, and may be confounded by population quality (Blanquart et al., 2013). A third, meta-population approach, has been suggested to be more adequate for rigorous testing of local adaptation: the "sympatric vs. allopatric" criterion, which compares the average fitness in sympatric combinations (populations at home site) and the average fitness of allopatric combinations (populations in foreign site). This method has been applied to investigate local adaptation in the four aforementioned alpine species in **Chapter 5**, **6**, and **7**.

Reciprocal transplantation experiments can than be coupled with molecular analysis, used to genotype the individuals from different populations (new microsatellite markers were developed **Chapter 8** and **9**), to compare phenotypic differentiation with molecular differentiation and infer patterns of adaptation.

Outline of the thesis

Chapter 1. General Introduction – this chapter.

Chapter 2. Lower plasticity exhibited by high- versus mid-elevation species in their phenological responses to manipulated temperature and drought

S. Gugger, H. Kesselring, J. Stöcklin, **E. Hamann***

*E. Hamann is the corresponding author and wrote the manuscript. The data is derived from S. Gugger's Master Thesis, supervised by E. Hamann.

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Reproduction is particularly challenging at high elevation, due to the short growing season and low temperatures, and requires fine-tuning to environmental cues. The aim of this study was to examine the shifts in reproductive phenology, the timing of life-history events, exhibited by high-elevation species in response to advanced spring temperatures and limited soil water availability.

For this purpose, we reciprocally transplanted 14 perennial herbaceous high elevation species to common gardens at 1000 and 2000 m.a.s.l that mimic prospective climates. A drought treatment was implemented to assess the combined effects of temperature and precipitation changes on the onset and duration of reproductive phenophases. This design was replicated with congeneric mid-elevation species to evaluate if mid- and high-elevation species harbor the same potential for plasticity in their reproductive phenology, which could be constrained for high elevation species by their specific adaptation to the alpine environment.

Chapter 3. Plant responses to simulated warming and drought: a comparative study of functional plasticity between congeneric mid and high elevation species

E. Hamann, H. Kesselring, J. Stöcklin

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DOI:10.1093/jpe/rtx023, available online at www.jpe.oxfordjournals.org

Alpine regions are frequently considered as being at risk from warming temperatures and drough. Phenotypic plasticity could help species limit the negative effects of environmental variations and buffer against climate change.

14 congeneric mid- and high elevation species were transplanted to two common gardens (1000 and 2000 m.a.s.l.) with differing watering regimes and we examined whether key functional plant traits, such as leaf traits and biomass allocation adjusted plastically to changes in temperature and soil water availability. A comparative approach between mid- and high-elevation species was used to infer on the consistency of species' responses to climate change and a phenotypic plasticity index was used to compare the degree of phenotypic plasticity between species' origin, to assess if high elevation species harbor the same potential for phenotypic plasticity as their lower elevation congeners.

Chapter 4. Past selection explains differentiation in flowering phenology of nearby population of a common alpine plant

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Alpine Botany (2015) 125: 113-124
DOI: 10.1007/s00035-015-0157-z, available online at www.springer.com

The timing of and relative investment in reproductive events are crucial fitness determinants for alpine plants, which have limited opportunities for reproduction in the col and short growing season at high elevations.
We used *Anthyllis vulneraria* to study whether flowering phenology and reproductive allocation have been under diversifying selection, and to assess genetic diversity and plastic responses to drought in these traits. Open-pollinated maternal families from three populations in each of two regions from the Swiss Alps with contrasting precipitation were grown in low and high soil moisture in a common garden. We measured onset, peak, and end of flowering, as well as vegetative and reproductive aboveground biomass. Population differentiation for each character (Q_{ST}) was compared to differentiation at neutral microsatellite loci (F_{ST}) to test for past selection.

Chapter 5. Evidence of local adaptation to fine- and coarse-grained environmental variability in *Poa alpina* in the Swiss Alps

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Journal of Ecology (2016) 104: 1627-1637
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In the Alpine landscape, characterized by high spatiotemporal heterogeneity, intraspecific plant variation is high and can arise from divergent selection leading to genetic differentiation among populations, or adaptive phenotypic plasticity. The relative importance of these processes is likely to be related to the spatial scale of environmental heterogeneity and gene flow among populations.
In this study we reciprocally transplanted the widespread alpine grass, *Poa alpina*, within and across regions in the Swiss Alps. Using fitness-related traits investigated across the sympatric vs. near- or far allopatric contrast, we infer on patterns of local adaptation across two spatial scales. Additionally, we measured specific leaf area to investigate potential selection on phenotypic plasticity. In parallel, all populations were genotyped with neutral microsatellite markers to assess molecular differentiation.

Chapter 6. High intrascpecific phenotypic variation, but little evidence of local adaptation in *Geum reptans* populations in the Central Swiss Alps

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Alpine Botany (2017) 127: 121-132
DOI: 10.1007/s00035-017-0185-y, available at www.springer.com

Intraspecific phenotypic variation is frequent in plant populations widespread across the heterogeneous and fragmented Alpine landscape. In this context, divergent selection can

lead to local adaptation, contingent however on several factors such as the spatial distance between populations, gene flow, and species' reproductive mode (i.e. clonality). Here, we reciprocally transplanted 3 populations of the high-alpine clonal *Geum reptans,* growing at close or far geographical distance from each other, and compared growth- and fitness-related traits across sympatric and near- or far-allopatric transplant combinations to investigate patterns of local adaptation. We further measured leaf morphology traits known to be particularly plastic in response to environmental variation. For all traits, we quantified the importance of genetic vs. environmental variation (i.e. phenotypic plasticity), and for leaf traits we assessed potential selection on mean trait value at field sites. Additionally, among and within population genetic differentiation was analyzed using microsatellite markers.

Chapter 7. Spatial patterns of local adaptation in two common herbs from the Central European Alps

H. Kesselring, J. Scheepens, **E. Hamann**, G. Armbruster, J. Stöcklin
In preparation for *Plant Ecology*

Spatially variable selection is considered to result in local adaptation. Yet the generality of local adaptation of populations remains debated, and we know little about the spatial patterns of local adaptation.
We conducted reciprocal transplantations among six populations each of two common and well-studied herbaceous plants, *Anthyllis vulneraria* and *Arabis alpina*. We measured aboveground biomass, reproductive allocation and flowering propensity to test for local adaptation at two spatial scales: within and between the Eastern and Western Swiss Alps. Additionally, populations were genotyped using microsatellite markers to assess neutral differentiation and historic inbreeding.

Chapter 8. Novel microsatellite markers for the high-alpine *Geum reptans*

E. Hamann, H. Kesselring, J. Stöcklin, G. F. J. Armbruster
Applications in Plant Sciences 2(6), 2014
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Geum reptans, a species that reproduces by outcrossing or by the formation of stolons, was genotyped to assess the genotypic differentiation between populations. For that purpose, novel microsatellite primers had to be developed for this species, which will be used in a study about local adaptation, phenotypic plasticity, and molecular differentiation of alpine plants.

Chapter 9. New microsatellite markers for Anthyllis vulneraria (Fabaceae), analyzed with Spreadex gel electrophoresis

H. Kesselring, **E. Hamann**, J. Stöcklin, G. F. J. Armbruster
Applications in Plant Sciences 1(12), 2013
DOI: 10.3732/apps.1300054, avaible at www.bioone.org

New microsatellite primers were developed for the diploid herb *Anthyllis vulneraria*. These primers will be used in upcoming studies focusing on random genetic variation, local adaptation, and phenotypic plasticity in alpine plants. Our preliminary results showed that the three studied alpine populations are predominantly outcrossing, but include variable levels of self-fertilization.

Chapter 10. General Discussion

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Chapter 2

Lower plasticity exhibited by high- versus mid-elevation species in their phenological responses to manipulated temperature and drought

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Lower plasticity exhibited by high- versus mid-elevation species in their phenological responses to manipulated temperature and drought

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Abstract

• Recent global changes, particularly warming and drought, have had worldwide repercussions on the timing of flowering events for many plant species. Phenological shifts have also been reported in alpine environments, where short growing seasons and low temperatures make reproduction particularly challenging, requiring fine-tuning to environmental cues. However, it remains unclear if species from such habitats, with their specific adaptations, harbour the same potential for phenological plasticity as species from less demanding habitats.

• Fourteen congeneric species pairs originating from mid and high elevation were reciprocally transplanted to common gardens at 1050 and 2000 m a.s.l. that mimic prospective climates and natural field conditions. A drought treatment was implemented to assess the combined effects of temperature and precipitation changes on the onset and duration of reproductive phenophases. A phenotypic plasticity index was calculated to evaluate if mid- and high-elevation species harbour the same potential for plasticity in reproductive phenology.

• Transplantations resulted in considerable shifts in reproductive phenology, with highly advanced initiation and shortened phenophases at the lower (and warmer) site for both mid- and high-elevation species. Drought stress amplified these responses and induced even further advances and shortening of phenophases, a response consistent with an 'escape strategy'. The observed phenological shifts were generally smaller in number of days for high-elevation species and resulted in a smaller phenotypic plasticity index, relative to their mid-elevation congeners.

• While mid- and high-elevation species seem to adequately shift their reproductive phenology to track ongoing climate changes, high-elevation species were less capable of doing so and appeared more genetically constrained to their specific adaptations to an extreme environment (i.e. a short, cold growing season).

Keywords: Climate change, flowering phenology, phenotypic plasticity, global warming, drought, common garden, mid-elevation and high-elevation species, Swiss Alps.

Introduction

In seasonal climates, the timing of flowering is crucial for plant reproductive success. Premature or late flowering can expose plants to adverse environmental conditions such as frost events (Inouye, 2008), can disrupt plant-pollinator interactions (Memmott et al., 2007) and can lead to failures in seed set or maturation. The timing of seasonal activities in plants has thus evolved to be triggered by reliable environmental cues such as date of snowmelt, photoperiod, temperature or soil moisture to guarantee reproductive success (Rathcke and Lacey, 1985). Recent global change has led to increased temperatures and to more frequent and more extreme floods and droughts in some areas (Hartmann et al., 2013) with repercussions on these environmental cues. Shifts in phenological events have been used as 'fingerprints' of ongoing climate change (Walther et al., 2002, Jentsch et al., 2009) and are well documented in numerous global-scale studies (Parmesan and Yohe, 2003, Peñuelas et al., 2004, Menzel et al., 2006, Cleland et al., 2007).

Phenotypic plasticity may play a crucial role in the short-term adjustment to novel conditions and can promote long-term adaptive evolution by buffering against rapid change (Price et al., 2003, Nicotra et al., 2010, Richter et al., 2012). Although a potential for rapid adaptive evolution in flowering phenology has been found (Franks et al., 2007, Haggerty and Galloway, 2011, Anderson et al., 2012) it remains unclear if natural selection can keep pace with the speed of ongoing changes (Visser, 2008, Shaw and Etterson, 2012). Alternatively, numerous plastic adjustments to current climate change such as advanced and accelerated phenophases in response to earlier snowmelt and spring warming have been documented worldwide (Abu-Asab et al., 2001, Fitter and Fitter, 2002, Cleland et al., 2007, Vitasse et al., 2013).

In Europe, springtime has advanced by 2.5 d per decade since the 1970s and delayed autumn events have led to an extension of the annual growing season (Menzel et al., 2006). Longer and warmer growing seasons could be associated with enhanced plant growth (Hudson et al., 2011), although limiting factors such as reduced water availability in summer could have negative effects. Indeed, summers in Switzerland have become drier over the past 30 years (Beniston et al., 1994, Kovats et al., 2014), and drought stress is known to influence plant growth, performance and reproductive success (Levitt, 1980) and is likely to also affect plant phenology (Peñuelas et al., 2004). While some studies report on advanced flowering dates in response to drought (Jentsch et al., 2009, Bernal et al., 2011, Franks, 2011) others found delayed flowering (Llorens and Peñuelas, 2005). Phenological responses to drought appear to be highly species specific (Bernal et al., 2011) as well as dependent upon the specific ecosystem (Peñuelas et al., 2004), and to follow complex spatiotemporal patterns (Peñuelas et al., 2004). Furthermore, little is known about the combined effect of warming and drought on flowering phenology (Dunne et al., 2003, Bloor et al., 2010).

In the Swiss Alps, the increase in temperature has been shown to be twice as high as that reported globally (Beniston et al., 1994), and summer droughts are predicted to become more frequent (Beniston et al., 1997, Kovats et al., 2014) making mountain biota in this region particularly exposed to climate change (Theurillat and Guisan, 2001, Körner, 2003).

For alpine plants, reproduction is especially challenging and the timing of flowering even more central to reproductive success as the timeframe for growth and reproduction becomes progressively shorter with increasing elevation (Billings and Mooney, 1968, Körner, 2003). Few studies have examined the effect of drought on the phenology of alpine vegetation and generally found no shifts (Bloor et al., 2010, Cornelius et al., 2013). However, advanced flowering was found when plants were grown in warmer conditions (Scheepens and Stöcklin, 2013, Frei et al., 2014a), and other studies with similar findings debated whether phenological shifts were triggered by higher air temperatures or advanced snowmelt (Price and Waser, 1998, Dunne et al., 2003, Cornelius et al., 2013).

Furthermore, photoperiod plays a key role in protecting plants from hazardous sprouting before the typical last date of severe spring frosts. Keller and Körner (2003) found that half of 23 study species were highly sensitive to photoperiod, and a later publication from Basler and Koerner (2012) specified that particularly late-successional species are photoperiod sensitive, and may not react to periods of earlier snowmelt or higher temperatures. This high level of adaptation to the particular alpine conditions raises the question of whether high-elevation species harbour the same potential for phenological plasticity as mid- elevation species. As high-elevation species are adapted to short growing seasons and have evolved to avoid frost damage, the onset of flowering phenology is likely to be genetically fixed (Keller and Körner, 2003), constraining their capacity to respond plastically to changes in external conditions. While Vitasse et al. (2013) found lower phenological plasticity in high-elevation deciduous tree species, a reciprocal transplant experiment with three grassland species revealed no difference in plasticity between low- and high-elevation populations (Frei et al., 2014a). However, to our knowledge no study has examined if mid- and high-elevation herbaceous species harbour the same potential for phenotypic plasticity in flowering phenology on a larger scale.

To examine how the combined effects of warming and drought affect the flowering phenology of mid- and high- elevation species as well as to examine whether phenotypic plasticity in flowering phenology differs between species origin, we reciprocally transplanted 14 congeneric pairs of herbaceous perennial mid- and high-elevation species between common gardens at 1050 and 2000 m a.s.l. Rain-shelters were used at each site to control the water input to our system to mimic severe drought events in summer. The study examined whether transplantation and drought events induced shifts in the flowering phenology of mid- and high-elevation species. Specifically, we tested the following expectations: (1) earlier onsets and expanded durations of phenophases at the lower (warmer) site taking advantage of a longer growing season, (2) delayed and shortened durations at the high-elevation site in accordance with later snowmelt and a shorter growing season, (3) earlier onsets and shortened durations of phenological stages in response to drought which acts to shorten the growing season, and (4) a lower phenological plasticity in high-elevation species, stemming from putative constrained adaptations to cold environments.

Material and Methods

Common gardens and study species

Two common gardens (Supplementary Data Fig. S1) were established in the Bernese Highlands in Switzerland, each accommodating four beddings delimited by a wooden frame (1 x 3 m). The high-elevation common garden is situated on the Schynige Platte (46° 39' 03.63'' N, 7° 54' 32.76'' E) at 2000 m a.s.l. on a southern slope. The snow-free period generally starts in June and lasts until October (approx. 150 d). The average annual temperature is 1° C and the average annual amount of precipitation is approx. 1600–2000 mm, of which half falls as snow (MeteoSwiss, 2014). The lower elevation common garden is situated in Zweilütschinen (46° 38' 26.55'' N, 7° 54 ' 15.20'' E). This was at 1050 m a.s.l. with a south/south-western slope. The snow-free period usually lasts from mid-April to December (approx. 250 d). The average annual temperature is 7.2° C and average annual precipitation is approx. 1100 mm, of which a quarter falls as snow (MeteoSwiss, 2014).

Twenty-eight perennial herbaceous species were included in this study, represented by 14 congeneric pairs of mid- and high- elevation species (Table 1). The species pairs were selected to cover a broad range of taxonomic groups and growth forms while avoiding an overlap in their altitudinal range of distribution. The ranges of mid-elevation species lie between approx. 300 and 1000 m.a.s.l, while the ranges of high-elevation species are mostly between approx. 1600 and 2400 m.a.s.l. (Table 1; Lauber and Wagner, 2001, Aeschimann et al., 2004). Seeds collected from flowers from wild populations were purchased from Swiss seed producers (Samen & Pflanzen AG Schutz, Filisur; UFA-Samen, fenaco Genossenschaft, Winterthur; Wildstaudengärtnerei, Eschenbach).

Experimental design

In spring 2012, seeds were germinated on moist blotting pa- per in the glasshouse of the Botanical Institute in Basel, Switzerland. Seedlings were individually transferred into multi-trays (4 cm diameter, 6 x 9 = 54 pots) filled with low-nutrient soil (Anzuchterde Ökohum, Herrenhof, Switzerland). In mid June, plants were brought outside in the garden of the Botanical Institute to allow acclimation to outdoor conditions. At the beginning of July, plants were transported to the common gardens and transplanted into bigger pots (11.5 x 11.5 x 21.5 cm) filled with the same potting soil. At each site, 12 individuals of each species were randomized in the beddings previously enriched with potting soil and sunk to one-third depth into the soil. This design was systematically replicated in the beddings receiving rain-shelters, resulting in an experiment including a total of 1344 individuals across both sites and treatments (12 replicates x 2 sites x 2 treatments x 28 species = 1344 individuals; Fig. S1). The rain-shelters were installed after a week of acclimation and consisted of a triangular aluminium frame covered by an UV-B-transmissible greenhouse film (Luminance AF Window, Folitec, Germany) with a base area of 2.4 x 3.0 m and a height of 1·2 m. The tunnel shape with large openings al- lowed for constant wind flow preventing warming beneath the shelters. To minimize edge effects, the sheltered base was larger than the central 1 x 2.5 -m area occupied by plants. To avoid lethal consequences of the drought treatment, a minimal water input was provided. Twenty liters of rainwater was distributed per bedding every 2 weeks (approx. 0.12 L per individual). Accordingly, the difference in water availability between the beddings with and without rain-shelter equals the

amount of precipitation. At the end of the first growing season, rain-shelters were removed and plants overwintered under snow.

Table 1 Overview of the congeneric pairs of mid- and high elevation species included in our study with their main range limits in the literature having only been given in terms of altitudinal zonations as defined for the European Alps by Lauber and Wagner (2001) and Aeschiman *et al*. (2004): "colline" = 300 m to 900 m; "montane" = 900 m to 1500 m; "subalpine" = 1600 m to 2300 m; "alpine" = 2300 m to 3000 m. "Mid-elevation" species mainly ranged from the colline to the lower montane zones, while "high elevation" species mainly ranged from the subalpine to the alpine zones.

Family	Mid elevation species	High elevation species
Lamiaceae	*Acinos arvensis* (Lam.) Dandy colline-montane	*Acinos alpinus* (L.) Moench subalpine
Poaceae	*Anthoxanthum odoratum* L. colline-alpine	*Anthoxanthum alpinum* Löve subalpine-alpine
Fabaceae	*Anthyllis vulneraria ssp. vulneraria* L. s.l. colline-montane	*Anthyllis vulneraria ssp. alpéstris* Schult subalpine-alpine
Brassicaceae	*Arabis hirsuta* L. colline-montane	*Arabis alpina* L. s.l. montane-alpine
Campanulaceae	*Campanula rotundifolia* L. colline-subalpine	*Campanula scheuchzeri* Vill. subalpine-alpine
Asteraceae	*Centaurea scabiosa* L. s.l. colline-montane	*Centaurea montana* L. montane-subalpine
Caryophyllaceae	*Dianthus deltoides* L. colline-montane	*Dianthus sylvestris* Wulfen colline-subalpine
Rosaceae	*Geum urbanum* L. colline-montane	*Geum montanum* L. subalpine-alpine
Fabaceae	*Lotus corniculatus* L. colline-subalpine	*Lotus alpinus* Ramond alpine
Fabaceae	*Onobrychis viccifolia* Scop. colline-montane	*Onobrychis montana* DC. subalpine
Poaceae	*Phleum phleoides* (L.) Karsten colline-montane	*Phleum alpinum* L. subalpine-alpine
Plantaginaceae	*Plantago lanceolata* L. colline-subalpine	*Plantago alpina* L. subalpine-alpine
Caryophyllaceae	*Silene vulgaris ssp. vulgaris* (Moench) Garcke s.l. colline-subalpine	*Silene vulgaris ssp. glareosa* (Jord.) Marsd.-Jon & Turill alpine
Fabaceae	*Trifolium pratense ssp. pratense* L. colline-subalpine	*Trifolium pratense ssp. nivale* (Koch) alpine

In Spring 2013, rain-shelters were reinstalled right after snowmelt (early May at the low common garden and mid-June at the high common garden) initiating the start of phenological recordings (plants did not reproduce in the first year). Air temperature was recorded hourly in each common garden and treatment at 0·5 m above the ground using sheltered data loggers (TidBit v.2 UTBI-001; Onset Computer Corp., Bourne, MA, USA). Similarly, light intensity loggers (Hobo pendant light data logger 64 K-UA-002-64; Onset Computer) were installed in each common garden at 1 m above the ground in both treat- ments. The drought treatment consisted of a minimal water input as in the previous year. Once a month, the volumetric soil moisture content (VSCM; m^3 m^{-3}) was measured randomly in 30 pots of each bedding with an HH2 Moisture Meter and a Theta Probe type ML2x (Delta-T Devices, Cambridge, UK).

Abiotic treatment effect

Averaged over the experimental period (May–October, Table 2), at the mid-elevation common garden, the daily temperature was 15.5° C in control beddings and 15.9° C in beddings topped by rain-shelters. In the high-elevation common garden, the average daily temperature was 11.2° C in control beddings and 11.4° C in beddings topped by rain-shelters. While there was a significant temperature difference between both common gardens, the rain-shelters increased the temperature at ground level only marginally by 0.25° C.

The recorded light intensity (measured in lux at 13:00 h) was higher at the high-elevation common garden and was significantly reduced by rain-shelters (Table 2). At both common gardens, the rain-shelters intercepted approx. 30 % of light but these values were not limiting for plant growth (see fig 11.11 in Körner, 2003).

VSMC (Table 2) differed significantly between the control and the drought treatment in both the common gardens ($W = 900$, $P = 10^{-4}$; $W = 844.5$, $P = 10^{-4}$, respectively). At the mid-elevation site, the average VSMC of control pots equaled 0.40 ± 0.08 m^3 m^{-3}, while dry pots had a VSMC of 0.06 ± 0.02 m^3 m^{-3}. At the high-elevation site, control pots had an average VSMC of 0.48 ± 0.1 m^3 m^{-3}, while dry pots had an average VSMC of 0.08 ± 0.02 m^3 m^{-3}.

Table 2: Mean temperature, light intensity and volumetric soil moisture content (VSMC) for each treatment averaged over the experimental period (May-September).

	Temprature (°C)	Light Intensity (lux)	VSMC (m^3 m^{-3})
Low site / Control	15.5	115323.5	0.4
Low site / Dry	15.9	84554.8	0.06
High site / Control	11.2	139846.9	0.48
High site / Dry	11.4	101209.8	0.08

Phenology monitoring

Phenological stages were defined after Price and Waser (1998), Dunne et al. (2003). Different stages were used for forbs and grasses to account for their morphological differences. Seven stages were defined for forbs: unopened buds, opened buds, opened flowers, old flowers, initiated fruits, enlarged fruits and dehisced fruit. For grasses, five stages were defined: beginning of heading, end of heading, exerted anthers or styles, dried and broken-off anthers/styles, and

disarticulated seeds.

All observed stages were recorded weekly per individual and when 50 % or more of the flowers or inflorescences were in a particular stage it was identified as dominant. Once all plants had completed their reproductive cycle and the growing season came to an end, all plants were harvested. Above-ground biomass was cut at soil level and individuals were stored in parchment bags and transported to the laboratory within 24 h, dried for 72 h at 80° C and weighed.

Phenological variables

Eight phenological variables were derived from the weekly recordings: onset of budding, onset of flowering, onset of fruiting, midpoint of flowering, duration of budding, duration of flowering, duration of fruiting and total duration of all three phenophases combined. Onset of budding, flowering and fruiting were defined as the date (day of the year) when the first bud, flower or fruit was observed. Midpoint of flowering was defined as the average date when opened flowers or exerted anthers/styles (for forbs and grasses, respectively) were dominant. The duration of a phenophase was defined as the number of days between the onset of said phenophase and the dominance of the following phenophase.

Phenotypic plasticity in flowering phenology

The degree of phenotypic plasticity in response to warming and drought was calculated as a phenotypic plasticity index (Pi_V) (Valladares et al., 2006). This index was calculated as the difference between the maximum and the minimum mean value of a given trait and species over all treatments divided by the maximum mean, which serves to standardize the index ranging from 0 (no plasticity) to 1 (maximum plasticity). Note that plasticity was considered at the species level rather than at the genotype level to compare the degree of plasticity between mid- and high-elevation species.

Statistical analysis

To test treatment effects, a linear mixed-effect model was used for all eight phenological variables. 'Elevation' (mid- or high-elevation site), 'drought' (control or drought treatment), 'origin' of species (mid-elevation or high-elevation) and their respective interactions were computed as fixed effects. To account for variances between species, they were nested in their respective genus and computed as random effects. The effects of 'elevation' and/or 'drought' indicate trait variation due to different environmental conditions (i.e. phenotypic plasticity), while the 'origin' of species effect indicates differences between mid- and high-elevation species. The interaction between 'origin' of species and 'elevation' and/or 'drought' indicates a difference in the responses to treatment conditions between mid- and high-elevation species. Aboveground dry mass was used as a covariate to correct for size effects on phenology, but was removed as it did not change the results or add value to the model. All linear mixed-effect models were implemented with the 'lmerTest' package for R software (Kuznetsova et al., 2013), based on type 3 errors and Satterthwaite approximation for denominator degrees of freedom. Post-hoc Tukey's HSD tests for multiple comparisons were performed using the 'multcomp' package (Hothorn et al., 2014) for R software.

To test for differences in the degree of phenotypic plasticity of flowering phenology between mid- and high-elevation species, the Pi_V calculated for each species was analysed with a paired Wilcoxon signed rank test. All the analyses were

performed in R version 3.0.2 software (R Development Core Team, 2013; https://www.r-project.org/).

Results

Many individuals died over winter, were subjected to herbivory or were not reproductive, leading to the total exclusion of four genera (Centaurea, Geum, Onobrychis and Trifolium) from the analysis. For the remaining species, an average of 8.3 replicates per treatment combination were included in the final analysis with a total of 667 individuals (i.e. 20 out of 28 initial species and approx. 50 % of the initial sample size). Mortality, however, was independent of species' origin and treatment combinations (Fisher's exact test for count data: $P = 0.85$). In 2013, the average temperature during the growing season differed by 4.4° C between common gardens and on average the drought treatment reduced VSMC by 0.37 m^3 m^{-3}. These changes in abiotic conditions induced highly species-specific shifts in the onsets and durations of phenophases but important patterns emerged when groups of mid- and high-elevation species were considered. To enhance clarity, we first report results from the control treatment, describing the shifts in reproductive phenology in response to temperature for mid- and high-elevation species and second drought effects.

Transplantation effect

The reciprocal transplantation of species to a warmer or colder prospective climate induced major shifts in the time of initiation and the duration of reproductive phenology. The onsets of budding, flowering and fruiting were always initiated earlier at the low-elevation site by at least a month, but mid- and high-elevation species differed in their response to transplantations. While the differences between mid- and high-elevation species in phenological onsets were not always revealed by post-hoc multiple comparisons (Fig. 1), they were highly significant overall, as indicated by the significant interaction between elevation and origin treatments (Table 3; budding $F = 7.64$, $P = 0.006$; flowering $F = 16.27$, $P < 10^{-4}$; fruiting $F = 15.48$, $P < 10^{-4}$, respectively).

Indeed, high-elevation species consistently initiated the onset of budding, flowering and fruiting earlier than mid-elevation species, and these differences were particularly pronounced at the high-elevation site (Table 3; $F = 7.64$, $P = 0.006$; $F = 16.27$, $P < 10^{-4}$; $F = 15.48$, $P < 10^{-4}$, respectively). High-elevation species started budding 8.4 ± 2.2 d earlier than mid-elevation species when grown at the high-elevation site and 5.5 ± 2.2 d earlier when grown at the mid-elevation site (Fig. 1, Table S1). High-elevation species also started flowering and fruiting earlier than mid-elevation species, with strongest responses at the high elevation-site (Fig. 1). The same was found for the midpoint of flowering, which was always reached earlier by high-elevation species relative to their lower elevation congeners, especially at the high-elevation site (Table 3; $F = 29.47$, $P < 10^{-4}$). Midpoint of flowering was recorded 12.9 ± 2.3 d earlier for high-elevation species grown at the high-elevation site and 5.7 ± 2.6 d earlier when grown at the mid-elevation site (Fig. 1, Table S1).

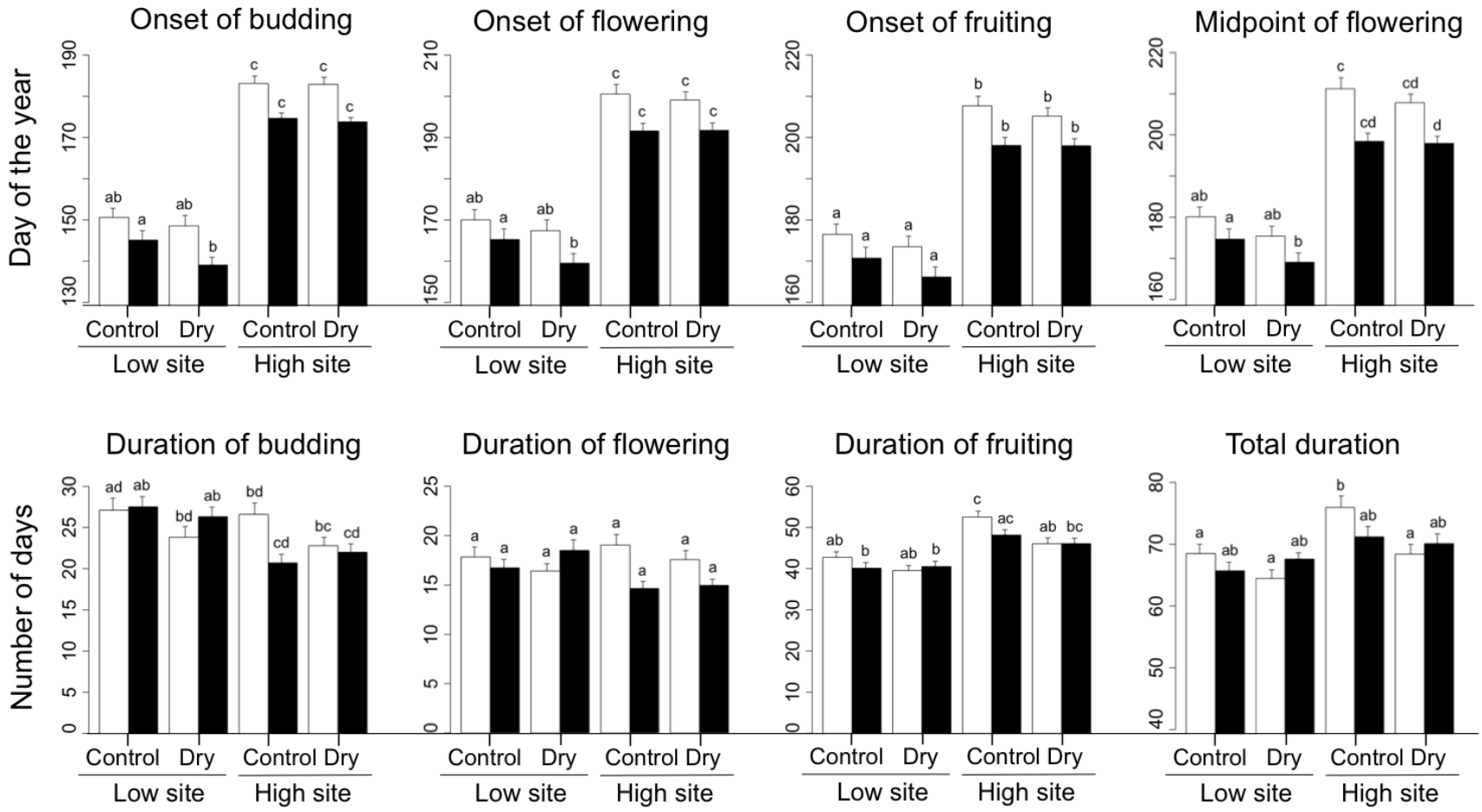

Fig. 1: Responses of mid elevation (white bars) and high elevation species (black bars) to transplantational elevation and drought treatment (mean ± s.e.) in onset, midpoint and duration of phenophases. The average onsets of budding, fruiting and flowering and the midpoint of flowering are shown in absolute days of the year, while the average durations of phenophases are shown in number of days. The letters above each bar represent the results of *post-hoc* Tukey tests for multiple comparisons. While they often provide detailed information about the differences between treatment combinations, some interactions between main effects are not revealed by the analysis, although they are significant on average in the more powerful ANOVA analysis.

Table 3: Linear-mixed effect model for the responses of onsets and durations of phenological stages to the elevation (mid elevation vs. high elevation site) and drought (control vs. dry) treatment, the origin of the species (mid elevation vs. high elevation species) and their respective interactions. Non-significant interactions were removed from the final model. The significant p-values are shown in bold.

	Onset of budding			Onset of flowering			Onset of fruiting			Midpoint of flowering		
	NumDf	F	P	*NumDf*	F	P	*NumDf*	F	P	*NumDf*	F	P
Elevation	1	1293.8	**$<10^{-4}$**	1	1206.9	**$<10^{-4}$**	1	1191.1	**$<10^{-4}$**	1	1099.4	**$<10^{-4}$**
Drought	1	3.53	0.06	1	3.20	0.07	1	3.53	0.06	1	8.80	**0.003**
Origin	1	2.62	0.14	1	2.97	0.12	1	3.57	0.10	1	4.30	0.07
Elevation : drought	1	7.55	**0.006**	1	7.15	**0.008**	1	3.92	**0.048**	1	6.51	**0.01**
Elevation : origin	1	7.64	**0.006**	1	16.27	**$<10^{-4}$**	1	15.48	**$<10^{-4}$**	1	29.47	**$<10^{-4}$**
Drought : origin	1	1.95	0.16	1	2.16	0.14	1	0.94	0.33	1	0.22	0.64
Elevation : drought : origin	1	0.15	0.70	1	0.83	0.36	1	0.49	0.49	1	0.22	0.64
	Duration of budding			Duration of flowering			Duration of fruiting			Total duration		
	NumDf	F	P	*NumDf*	F	P	*NumDf*	F	P	*NumDf*	F	P
Elevation	1	37.63	**$<10^{-4}$**	1	2.29	0.13	1	44.87	**$<10^{-4}$**	1	11.53	**0.0007**
Drought	1	3.96	**0.047**	1	0.04	0.83	1	15.94	**$<10^{-4}$**	1	11.39	**0.0008**
Origin	1	0.06	0.81	1	1.80	0.22	1	0.22	0.65	1	0.00	0.95
Elevation : drought	1	0.06	0.81	1	0.15	0.70	1	3.45	0.06	1	4.80	**0.03**
Elevation : origin	1	6.03	**0.01**	1	3.73	0.54	1	0.00	1.00	1	0.48	0.49
Drought : origin	1	2.63	0.11	1	3.09	0.08	1	6.27	**0.01**	1	6.43	**0.01**
Elevation : drought : origin	1	1.61	0.21	1	0.75	0.39	1	0.03	0.85	1	0.25	0.62

* ANOVA was calculated on a total of 667 individuals including 20 species with an average number of 8.3 ± 2.85 replicates per treatment combination.

Furthermore, differences in responses between mid- and high-elevation species are also revealed in the fact that advancement of the onset of phenophases in response to transplantation between sites was consistently greater for mid-elevation species relative to high-elevation species (again, not revealed by post-hoc tests). For mid-elevation species, the onset of budding at the mid- and the high-elevation sites differed by 32.5 ± 2.0 d, whereas for high-elevation species the difference was less (29.5 ± 2.1 d; Fig. 1). Similar trends were found for the onset of flowering and fruiting. The differences in responses between mid- and high-elevation species were particularly pronounced for advancement in midpoint of flowering. Going from the 2000 -m site down to the 1050 -m site, mid-elevation species advanced midpoint of flowering by 31.2 ± 2.4 d (Table 3; $F = 29.47$, $P < 10^{-4}$), whereas high-elevation species advanced this stage by only 23.7 ± 2.2 d (Fig. 1, Table S1).

The duration of phenophases responded to transplantations, with the exception of the duration of flowering (Table 3). A significant interaction between elevation and origin was found for the duration of budding (Table 3; $F = 6.03$, $P = 0.01$), indicating a difference in response between mid- and high-elevation species. The duration of budding was generally shortened at the high-elevation site compared with the mid-elevation site, but this was significant only for high-elevation species, for which a contraction of 6.8 ± 1.1 d was recorded (Fig. 1, Table S1). For mid-elevation species, by contrast, this contraction was only of 0.5 ± 1.4 d. The duration of fruiting was significantly shorter at the mid-elevation site for both mid- and high-elevation species (Table 3; $F = 44.87$, $P < 10^{-4}$). The maturation of fruits took 9.8 ± 1.3 d less at the mid-elevation site compared to the high-elevation site for mid-elevation species, and 8.1 ± 1.3 d less for high-elevation species (Fig. 1, Table S1).

Note that mid-elevation species had a particularly long duration of fruiting when grown at high elevation (Fig. 1). The total duration of reproductive phenology was also shortened at the mid-elevation site. However, this effect was only significant for mid-elevation species, which had a 7.5 ± 1.6-d shorter duration of reproduction when grown at the lower site. The effects of transplantation on the total duration of reproductive phenology were similar to those on the duration of fruiting (Fig. 1), reflecting that this last stage was proportionally the longest.

Finally, it is important to note that at the mid-elevation common garden, all reproductive individuals from mid- and high-elevation species reached the final fruit maturation stage (defined as 50% or more flowers of one individual having reached stage 7: dehisced fruits for forbs and stage 5: disarticulated seeds for grasses). At the high-elevation common garden, 99% of high-elevation species finished fruit maturation but only 85% of mid-elevation individuals reached the final fruit maturation stage before final harvest.

Drought effect

Drought had a tendency to advance phenophases, but had the greatest effect at the low-elevation site. Drought consistently led to smaller advancement of phenophases than did transplantation to the warmer site (Fig. 1). Effects of drought on onset of budding, flowering, fruiting and midpoint of flowering varied depending on whether plants were grown at the mid- or high-elevation sites, as indicated by a significant interaction between elevation and drought (Table 3; ExD for budding $P = 0.006$;

flowering P = 0.008; fruiting P = 0.048; and flowering mid-point P = 0.01). Drought initiated earlier phenophases at both sites but this effect was significant only at the mid-elevation site for high-elevation species (Fig. 1). At the mid-elevation site, drought-stressed high-elevation species initiated budding 6.1 ± 2.1 d earlier than individuals under control conditions, while drought-stressed mid-elevation species started budding only 2.1 ± 2.3 d earlier. In contrast, at high elevation, the onset of budding was only marginally advanced in the drought treatment, namely by 0.2 ± 1.7 d for mid-elevation species and by 0.9 ± 1.1 d for high-elevation species (Fig. 1, Table S1). The same results were found for the onset of flowering and fruiting, and for the midpoint of flowering (Table 3), although the difference between mid- and high-elevation species was not revealed by post-hoc comparisons for the onset of flowering (Fig. 1).

The durations of phenophases were unequally affected by drought and only the duration of flowering did not change in response to drought (Table 3, Fig. 1). The duration of budding was significantly shorter on average under dry conditions (Table 3; F = 3.96, P = 0.047), but this was not revealed by the post-hoc multiple comparisons (Fig. 1). For mid-elevation species, drought reduced the duration of budding by 3.3 ± 1.3 d at the mid-elevation site, and by 3.8 ± 1.2 at the high-elevation site. This effect was less pronounced and less consistent in high-elevation species (Fig. 1, Table S1).

While the duration of fruiting was also generally shortened by drought at the lower site, a significant interaction between drought and origin was found (Table 3; $F = 6.27$, $P = 0.01$) meaning that mid- and high-elevation species responded differently to the drought treatment. At the lower site, drought affected the duration of fruiting only marginally for mid- and high-elevation species (Fig. 1). By contrast, at the high-elevation site, the duration of fruiting was significantly shortened by 6.4 ± 1.4 d for mid-elevation species under drought stress but only marginally by 2.1 ± 1.3 d for high-elevation species (Fig. 1, Table S1).

For the total duration, a significant interaction was found between drought and elevation, as well as between drought and origin (Table 3, $F = 4.8$, $P = 0.03$; $F = 6.3$, $P = 0.01$, respectively). Drought-induced shifts in the total duration of reproductive phenology were more pronounced at the high-elevation site than at the mid-elevation site and in mid-elevation species compared to high-elevation species. Drought significantly shortened total duration of reproduction for mid-elevation species when growing at the high-elevation site, namely by 7.5 ± 1.7 d, however this effect was only marginal for mid-elevation species when grown at the lower elevation site and for high-elevation species at both sites (Fig. 1).

Pi_v of mid- and high-elevation species

A significant difference in the Pi_v was found for the midpoint of flowering (Fig. 2; V = 40, P = 0.03). Mid-elevation species had a greater Pi_v than high-elevation species, indicating that the shift in midpoint of flowering in response to elevation and drought was greater for mid-elevation species than for high-elevation species (0.19 ± 0.04 and 0.15 ± 0.05, respectively). These results are consistent with the previously reported shifts in number of days. Furthermore, as the Pi_v of mid-elevation species was systematically greater (Fig. 2) we also compared the mean Pi_v across all traits between mid- and high-elevation species and found a significantly higher mean value for mid-elevation species (Pi_{vMid}

= 0.31 ± 0.1, Pi_{vHigh} = 0.26 ± 0.1; V = 36, P = 0.01). This overall result indicates that mid-elevation species tended to have a greater degree of phenotypic plasticity in their reproductive phenology than high-elevation species and hence a greater capacity to adjust these traits to environmental changes in temperature and water availability.

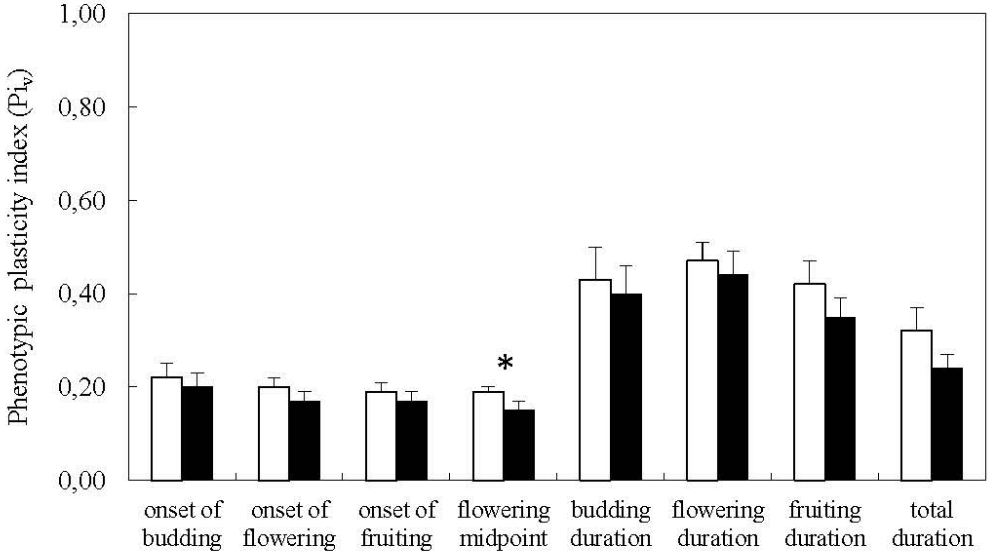

Fig. 2: Phenotypic Plasticity Index (Pi_v) of mid elevation (white bars) and high elevation species (black bars) calculated across all treatments for onsets and durations of phenophases. The error bars denote s.e. *, P<0.05.

Discussion

Responses to transplantation and drought

Transplantation of high-elevation species to a site with earlier springtime resulted in advanced onset of reproductive phenology, an overall pattern in agreement with existing literature (Price and Waser, 1998, Dunne et al., 2003, Scheepens and Stöcklin, 2013). Mid- and high-elevation species initiated all reproductive phenophases approx. 1 month earlier at the lower elevation site, indicating the important role of temperature for phenophases. Interestingly, high-elevation species initiated budding prior to mid-elevation species at both sites, on average by 10 d at the high site and by 5 d at the low site. In contrast, other studies found that reproduction was always initiated first by low-elevation populations at the low-elevation sites (Haggerty and Galloway, 2011, Frei et al., 2014a, Frei et al., 2014b). However, in those studies, experimental gardens were situated at lower elevations relative to our study sites (at 514 and 600 m compared with ours at 1050 m). This resulted in high-elevation populations in prior studies being exposed earlier in the year to days with higher temperatures, yet relatively shorter photoperiods than in our study, and may have driven the observed differences in results among our studies. As photoperiod is also a fundamental cue for a frost risk-free initiation of growth and reproduction for some alpine plants (Keller and Körner, 2003, Körner, 2003, Basler and Koerner, 2012), it is likely that high-elevation populations in the prior studies waited for days with a sufficiently long photoperiod and did not rely solely on temperature to initiate reproduction. However, in our study, photoperiod was similar between both common gardens at the

time of reproductive onset. Hence, advanced initiation of reproductive phenology in high-elevation species at both of our study sites probably reflects other adaptations to cold climates and short growing seasons, for example low growing degree day requirements (Haggerty and Galloway, 2011) (Haggerty and Galloway, 2011) and preformation of buds (Sørensen, 1941, Billings and Mooney, 1968, Bliss, 1971).

At the mid-elevation site, most phenophases were shortened, which is in agreement with previous studies (Sherry et al., 2007, Post et al., 2008, Steltzer and Post, 2009). However, the duration of budding was longer at the lower, warmer site relative to the high-elevation site, which highlights the contrasting effects of warming on individual phenophases (Post et al., 2008, Haggerty and Galloway, 2011, Cornelius et al., 2013). Contracted phenophases have generally been explained as resulting from increased developmental rates in warm conditions (Sherry et al., 2007, Haggerty and Galloway, 2011). Alternatively, extended reproductive durations are often linked to an expanded growing season (Dunne et al., 2003). In our study, the average daily temperature during the budding phase was higher at the mid- than at the high-elevation site (14.1 and 12.3° C, respectively). Thus, temperature alone cannot explain expanded budding duration, which is in contradiction with fast developmental rates expected under warm conditions. This result might be related mainly to the fact that high-elevation species significantly contracted this phenophase at high-elevation sites (Fig. 1) to guarantee sufficient time for flowering and fruit maturation, but it is also possible that plants tried to take advantage of a longer growing season at the lower site with advanced spring. The duration of fruiting was, however, highly accelerated for both groups of species by fast maturation rates under higher temperatures. As this last stage was proportionally the longest it resulted in a shorter total reproductive duration at the low-elevation site, which suggests that plants were not able to consistently prolong their reproductive cycle to take advantage of a longer growing season.

Limited water availability had considerable effects on plant reproductive phenology but drought-induced shifts were less extensive than those in response to temperature changes (shifts in the order of magnitude of a few days against a month, respectively). However, when drought stress was combined with higher temperatures, it generally emphasized the responses of species and consistently led to further advancements and shortenings of phenophases for mid-elevation species in responses to drought. This result is in line with a 4-d advancement in mid-flowering date recorded after a simulated drought in Central Europe (Jentsch et al., 2009) and with a study which revealed that an 'escape strategy' inducing earlier flowering was selected for in *Brassica rapa* following a natural drought (Franks et al., 2007, Franks, 2011). In our study, species responded to drought by plastic shifts congruent with such an 'escape strategy'. When the growing season is shortened by drought, plants with late reproductive initiations might be unable to mature seeds before conditions become lethal. Hence, when water availability is limited, a shift towards rapid development and maturation of flowers is advantageous and allows the maintenance of reproductive success (Vasek and Sauer, 1971, Franks, 2011).

Mid- and high-elevation species generally advanced phenophases in response to drought but changes in the duration of phenophases were less pronounced in high-elevation species. While the total duration

of reproductive phenology was mainly shortened for high-elevation species, a slight extension of budding and of fruiting was recorded at high- and mid-elevation sites, respectively. This result highlights the divergent effects of drought on certain phenophases (Peñuelas et al., 2004, Llorens and Peñuelas, 2005). In our case, high-elevation species are normally less exposed to drought periods than their congeners from lower elevations (Vasek and Sauer, 1971). Precipitation tends to increase with elevation and evapo-transpiration tends to decrease with elevation. Accordingly soil moisture availability generally increases with elevation (Körner, 2003). Consequently, the inconsistent responses of high-elevation species to drought at both sites suggest that although high-elevation species also tended towards an 'escape strategy' when facing drought, they might be less efficient in doing so then their mid-elevation congeners.

Constrained degree of phenotypic plasticity in high-elevation species

In line with our hypothesis, the differences between herbaceous mid- and high-elevation species affected their potential for phenological plasticity, as previously found for low- and high-elevation populations of deciduous tree species (Vitasse et al., 2013). Our results revealed that herbaceous high-elevation species tended to have a lower Pi_V than mid-elevation species for flowering phenology even though this difference was only significant for the midpoint of flowering and when averaged over all phonological variables. Nevertheless, both mid- and high-elevation species showed a notable capacity of tracking environmental changes through phenological shifts while maintaining a high performance. It is particularly interesting that high-elevation species were found to have a lower Pi_V specifically for the midpoint of flowering. The exact timing of flowering might be the most crucial phenophase for successful reproduction. The timing of flowering is even more crucial in cold environments, where short growing seasons (Billings and Mooney, 1968, Körner, 2003) and adverse conditions such as frost events (Inouye, 2008) pose additional challenges to reproductive success. Consequently, strong directional selection decreasing temperature sensitivity and increasing photoperiodic control (Basler and Koerner, 2012, Vitasse and Basler, 2013) may have shaped the evolution of reproductive phenology of high- elevation species to coincide with favourable environmental conditions, presumably contributing to local adaptation in heterogeneous landscapes (Hall and Willis, 2006, Verhoeven et al., 2008, Anderson et al., 2011).

The selective pressures controlling timing of reproduction become increasingly strong with elevation and thus we hypothesize that the difference in phenological plasticity would have been more pronounced if more strictly alpine species, from above treeline-elevation, had been chosen. This would have provided a more extreme contrast with congeneric mid- elevation species. Here, our results indicate that adaptation to short growing seasons in the alpine environment limits the potential for phenotypic plasticity in the reproductive phenology of high-elevation species in response to environmental changes, leading to a higher genetic canalization of the timing of peak flowering (Price et al., 2003, Pigliucci et al., 2006, Ghalambor et al., 2007).

Consequences of phenological shifts

For high-elevation species, transplantation to a lower elevation resulted in advanced phenophases, suggesting adaptive tracking of an advanced growing season (Cleland et al., 2012). However, higher temperatures also accelerated developmental rates and led to shortened phenophases, indicating that high-elevation plants were unable to take advantage of a longer growing season. Furthermore, in advanced growing seasons, the time frame for resource acquisition is abbreviated before environmental cues initiate reproduction. Consequently, advanced flowering could potentially lead to decreased fitness (Post et al., 2008, Scheepens and Stöcklin, 2013).

Alternatively, for mid-elevation species, the upward transplantation resulted in delayed initiation and prolonged phenophases. While the later initiation of reproduction at the higher site might be adaptive, the prolonged phenophases suggest an entirely passive response to slower developmental rates in cold conditions (Sherry et al., 2007). At the final harvest in late autumn 15 % of mid-elevation plants had not yet started to disperse their seeds and we estimate that in total approx. 30 % of flowers from mid-elevation species would not have completed fruit maturation (E. Hamann, pers. obs.). A prolonged reproductive period of upward migrated mid-elevation species could thus have fitness costs if associated with uncompleted seed maturation before winter fall.

Limited water availability advanced and shortened phenophases, a result congruent with the aforementioned 'escape strategy' limiting the negative impact of drought stress on plant fitness (Franks, 2011). However, drought-induced phenological shifts were greater for mid-elevation species, suggesting that they were more capable of adopting an efficient 'escape strategy' than their high-elevation congeners. Phenotypic plasticity has been suggested to be adaptive only when the environmental fluctuations experienced by populations do not fall outside their native range (Ghalambor et al., 2007). While mid-elevation species are frequently exposed to dry summer periods, high-elevation species have rarely experienced such environmental conditions in the past (Körner, 2003), which could explain why they were unable to produce an 'escape strategy' as efficient as their mid-elevation congeners.

We conclude that while the direction of plastic responses in reproductive phenology tended to track environmental changes, adaptation of species to their native range seem to constrain adaptive plasticity in novel conditions and could potentially lead to maladaptive responses (Ghalambor et al., 2007).

Supplementary Data

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Figure S1: schematic overview of the experimental design. Table S1: average day of the year of initiation and midpoint of phenological stages, and durations of phenophases reported for mid- and high-elevation species and treatment combinations.

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Supplementary Data

Table S1: Average day of the year (± SE) of initiations and midpoint of phenological stages, and durations (in number of days) of phenophases reported for the species groups (lowland and highland) and treatment combinations: A (low site/control), B (low site/dry), C (high site/control), D (high site/dry).

	Treatment	Lowland species	Highland species		Treatment	Lowland species	Highland species
Onset of budding	A	150.6 ± 2.2	145.1 ± 2.3	Duration of budding	A	27.1 ± 1.5	27.5 ± 1.3
	B	148.5 ± 2.6	139.0 ± 1.9		B	23.8 ± 1.3	26.2 ± 1.2
	C	183.1 ± 1.8	174.7 ± 1.3		C	26.6 ± 1.4	20.7 ± 1.0
	D	182.9 ± 1.7	173.8 ± 1.0		D	22.8 ± 1.0	22.0 ± 1.0
Onset of flowering	A	170.0 ± 2.5	165.2 ± 2.6	Duration of flowering	A	17.8 ± 1.0	16.7 ± 0.9
	B	167.4 ± 2.6	159.5 ± 2.3		B	16.4 ± 0.7	18.5 ± 1.1
	C	200.6 ± 2.3	191.6 ± 1.9		C	19.0 ± 1.1	14.7 ± 0.7
	D	199.1 ± 2.0	191.8 ± 1.8		D	17.6 ± 0.9	14.9 ± 0.6
Onset of fruiting	A	176.5 ± 2.5	170.7 ± 2.7	Duration of fruiting	A	42.7 ± 1.4	40.1 ± 1.4
	B	173.4 ± 2.6	166.1 ± 2.4		B	39.5 ± 1.2	40.4 ± 1.3
	C	207.7 ± 2.3	198.1 ± 1.9		C	52.5 ± 1.4	48.2 ± 1.3
	D	205.2 ± 2.1	198.0 ± 1.7		D	46.1 ± 1.4	46.1 ± 1.3
Midpoint of flowering	A	180.1 ± 2.4	174.7 ± 2.5	Total duration	A	68.4 ± 1.5	65.7 ± 1.4
	B	175.4 ± 2.4	169.0 ± 2.3		B	64.4 ± 1.4	67.6 ± 1.0
	C	211.3 ± 2.5	198.4 ± 2.0		C	75.9 ± 1.9	71.2 ± 1.7
	D	207.9 ± 2.1	197.9 ± 1.8		D	68.4 ± 1.6	70.1 ± 1.6

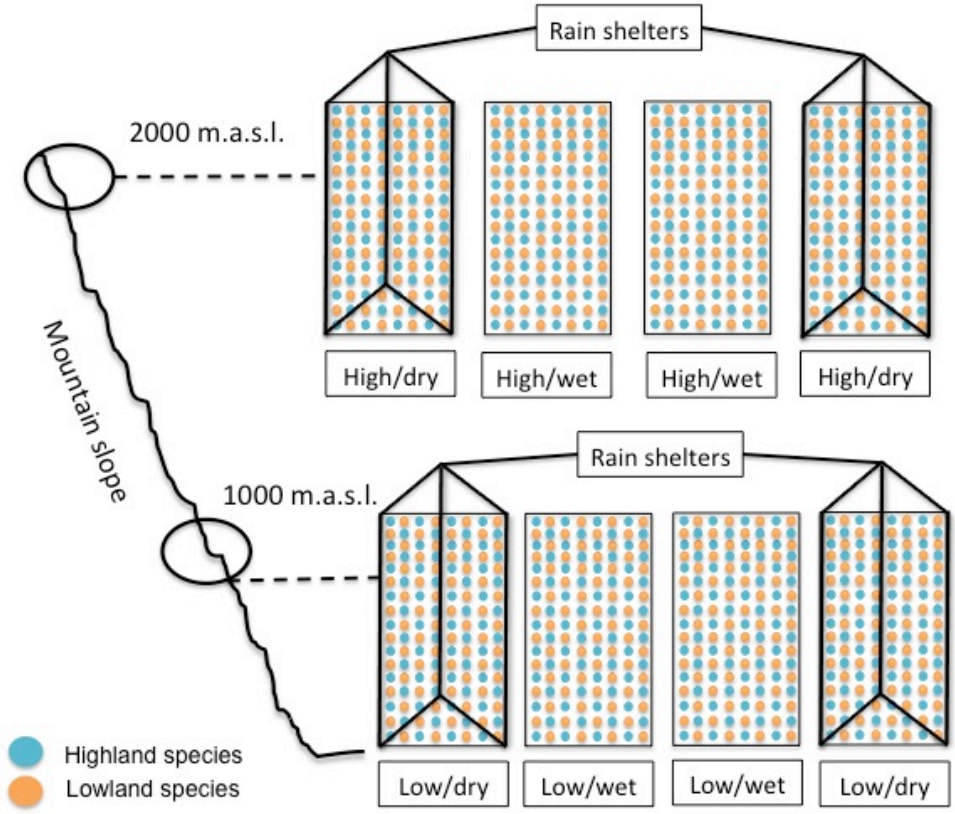

Fig. S1: Schematic overview of the experimental design.

Chapter 3

Plant responses to simulated warming and drought: a comparative study of functional plasticity between congeneric mid and high elevation species

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Responses of key plant traits to experimental warming and drought: a comparative study of functional plasticity between congeneric mid- and high elevation species

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Abstract

• Effects of climate change, especially changes in temperatures and precipitation patterns, are particularly pronounced in alpine regions. In response, plants may exhibit phenotypic plasticity in key functional traits allowing short-term adjustment to novel conditions. However, little is known about the degree of phenotypic plasticity of high elevation species relative to mid elevation congeners.

• We transplanted 14 herbaceous perennial species from high elevation into two common gardens (1050 and 2000 m.a.s.l.) in the Swiss Alps, and we examined plastic responses in key functional traits to changes in temperature and soil water availability. This design was replicated with 14 congeneric species from mid elevation to assess if the degree of phenotypic plasticity differs between mid and high elevation species. Survival was assessed across two growing seasons, while aboveground biomass and specific leaf area (SLA) were measured after the first growing season, and biomass allocation to belowground and reproductive structures after the second. Moreover, a phenotypic plasticity index was calculated for the functional traits to compare the degree of plasticity between mid and high elevation species.

• Aboveground biomass was higher in mid elevation species relative to high elevation congeners in all treatments, yet decreased for both with elevation and drought. Similarly, SLA decreased with elevation and drought. Root mass fraction (RMF) was generally higher in high elevation species, and decreased with drought at the lower site. Drought increased the allocation to reproductive structures, especially when plants were grown at their elevation of origin. Interestingly no difference was found in the degree of phenotypic plasticity averaged across mid and high elevation species for any of the studied functional traits.

• These results indicate that phenotypic plasticity in the focal traits did not depend on the elevation of origin of the species. Plasticity was not related to environmental heterogeneity, nor constrained by selective pressures at high elevation. However, both species groups showed a remarkable capacity for short-term acclimation to a prospective climate through rapid adjustments in key functional traits.

Keywords: Biomass allocation, Common garden, Climate change, Perennial herbaceous species, Phenotypic plasticity, SLA, Swiss Alps, Transplant experiment

Introduction

In the late nineteenth century, pioneer experimental botanists began using transplantation experiments along elevational gradients to investigate the degree of transformation of plants in novel environments (Kerner 1869, Bonnier 1890, ; reviewed in Briggs *et al.* 1997). Soon after, the genetic component of ecotypic differentiation of plants from different elevations was recognized by Clausen *et al.* (1941), along with the possibility that plants might change their phenotype depending on a given environment. This particular finding, later termed phenotypic plasticity, has received growing attention in the past decades (Bradshaw 1965, Schlichting 1986, Sultan 1987, Thompson 1991), and the current interest results in part from an urgency to predict species responses to global change (Valladares *et al.* 2006).

In Europe, increasing temperatures and changes in precipitation patterns have been reported by the IPCC (Hartmann *et al.* 2013, Kovats *et al.* 2014), and it has been suggested that the effects of global change are proportionally more important at high elevation (Beniston *et al.* 1997). Indeed, in alpine regions, the amplitude of temperature changes during the past decades is greater than globally observed changes (Beniston *et al.* 1994), and summer droughts are predicted to become more frequent (Kovats *et al.* 2014), leaving mountain biota particularly vulnerable to climate change (Theurillat *et al.* 2001, Körner 2003). In this context, phenotypic plasticity may play a crucial role in the short-term adjustment to novel conditions and could promote long-term adaptive evolution by buffering against rapid change (Price *et al.* 2003, Nicotra *et al.* 2010, Richter *et al.* 2012).

A number of studies have investigated shifts in plant traits in response to climate change. While modifications in flowering phenology are probably the best documented worldwide (Parmesan *et al.* 2003), adjustments in other key plant functional traits have also been reported in response to changes in temperature and soil water availability. Leaf traits and particularly specific leaf area (SLA) are considered as most informative (Wright *et al.* 2004, Scheepens *et al.* 2010), as SLA is an indicator of relative growth rate, stress tolerance and leaf longevity (Lavorel *et al.* 2002, Atkin *et al.* 2006, Poorter *et al.* 2009). SLA has been shown to strongly correlate with temperature, irradiance and soil water availability (Poorter *et al.* 2009), and generally decreases with increasing elevation (Körner 2003, Ma *et al.* 2010, Scheepens *et al.* 2010), and with reduced soil water availability (Poorter *et al.* 2009). SLA is a highly plastic trait, which adjusts rapidly to changing environmental conditions (Scheepens *et al.* 2010).

Increasing elevation and decreasing soil water availability are important factors limiting plant productivity. Indeed, aboveground biomass generally decreases with increasing elevation and drought (Lambers *et al.* 1998, Körner 2003). More specifically, the allocation of biomass to different plant organs differs along elevational and soil moisture gradients. Plant growth theory predicts that plants from stress dominated and cold habitats allocate a high portion of dry matter to belowground organs thereby increasing survival (Bloom *et al.* 1985, Grime 2001). Indeed, Körner *et al.* (1987) showed in an extensive study on 49 perennial herbaceous species, that high elevation plants allocate more dry matter to roots, especially fine roots, than typical lowland plants, and these results were

generally corroborated since (Prock *et al.* 1996, Ma *et al.* 2010, Poorter *et al.* 2012a). Similarly, in the context of drought stress, greater proportional root biomass presumably increases the uptake surface area and thus the water acquisition potential (Heschel *et al.* 2004, Pang *et al.* 2011, Huang *et al.* 2013). However, increased allocation to belowground structures may come at the expense of allocation to reproductive structures and/or photosynthetic organs such as leaves, and a trade-off between these structures has been found in several studies (Körner *et al.* 1987, Prock *et al.* 1996, Ma *et al.* 2010). Furthermore, when comparing high and low elevation species, it was found that high elevation species allocated three times more of their aboveground biomass specifically to floral structures (Fabbro *et al.* 2004), indicating a clear prioritisation of reproduction over growth. Similarly, under drought stress, trade-offs at the expense of reproductive structures have also been found (Huang *et al.* 2013). However, another study showed that two out of 11 alpine species had a higher reproductive biomass when grown under drought (Peterson *et al.* 1982), indicating that investment in sexual reproduction can be favoured in some species under drought stress or competition (Rautiainen *et al.* 2004).

Although a number of studies have examined the effects of warming and drought on plant traits (Arft *et al.* 1999, Heschel *et al.* 2004, Atkin *et al.* 2006, Gilgen *et al.* 2009), only few have studied the effects of these factors in combination, and simultaneously on multiple herbaceous species (Cleland *et al.* 2006, Bloor *et al.* 2010). Furthermore, to this day, we know of only two studies, which have used a comparative approach to examine if species or populations growing at high elevation harbour the same potential for phenotypic plasticity as their counterparts growing at lower elevation. While a reciprocal transplant experiment with three grassland species revealed no difference in the plasticity of growth, phenology and leaf traits between low and high elevation populations (Frei *et al.* 2014a), Vitasse *et al.* (2013) found lower phenological plasticity in high elevation deciduous tree species. Theory predicts that phenotypic plasticity is advantageous in spatially and temporally heterogeneous environments (Via *et al.* 1985, Alpert *et al.* 2002, van Kleunen *et al.* 2005). One could hypothesise that high elevation species, adapted to habitats with great spatial and temporal heterogeneity (Scherrer *et al.* 2011) might display greater plasticity in response to environmental variation than plants from lower more homogeneous sites. On the other hand, high elevation species have evolved under strong selective pressures, imposing directional or stabilizing selection on plant traits, and thereby constraining their capacity to respond plastically to changes in external conditions (Vitasse *et al.* 2013). In a parallel study, we found lower plasticity in the flowering phenology of high elevation species, which are probably constrained by canalized selection for rapid flowering after snowmelt in regard of the short growing season at high elevation (Gugger *et al.* 2015). In the traits studied here, we expect the opposite because high plasticity in SLA and biomass allocation might be advantageous in a highly heterogeneous environment such as the alpine habitat.

Here, we examine the combined effects of warming and drought on specific plant traits, known to be particularly plastic to these environmental factors (i.e. SLA, biomass allocation). We reciprocally transplanted 14 congeneric pairs of herbaceous perennial species originating from mid and high elevation sites in the Swiss Alps to common

gardens differing c. 1000 m in elevation to mimic changes in temperature, and installed rain-shelters to control soil water availability. Our factorial design allows to quantify the effects of simultaneous warming and drought on key plant functional traits and to test for differences in direction and magnitude of plastic responses between mid and high elevation species. Specifically, we expect: (1) plant productivity to decrease with elevation and drought (2) specific leaf area to decrease with increasing elevation and drought 3) allocation to belowground and reproductive structures to increase with drought and elevation, and to be proportionately greater in high elevation species (4) the degree of phenotypic plasticity to be higher in high elevation species relative to congeneric mid elevation species, resulting from adaptation to high environmental heterogeneity at high elevation.

Materials and methods

Common gardens and study species

Common gardens, with four plant beds each, were established at 1050 and 2000 m.a.s.l on the same mountain of the Bernese Highland in Switzerland. The difference in elevation between the common gardens entails for an annual mean air temperature difference of 5-6°C (Körner 2003), which mimics extreme warming scenarios of the IPCC by 2100 (Kovats *et al.* 2014). Specific site location and abiotic conditions have previously been described in a related paper (Gugger *et al.* 2015).

14 species pairs of congeneric perennial herbs naturally growing in the region and originating from mid and high elevations were selected for this study (Table 1), covering a broad taxonomic and growth form range. Mid elevation species were selected from elevations between c. 300 and 1000 m.a.s.l and high elevation species between c.1600 and 2400 m.a.s.l (Lauber *et al.* 2001, Aeschimann *et al.* 2004), as to avoid an overlap in their altitudinal range of distribution (see details in Table 1 in Gugger *et al.* 2015). Seed mixes, originally collected from wild flower populations from the aforementioned distributional ranges and then proliferated in gardens for two years, were purchased from Swiss seed producers (Samen und Pflanzen AG Schutz, Filisur; UFA-Samen, fenaco Genossenschaft, Winterthur; Wildstaudengärtnerei, Eschenbach).

Experimental design

For a detailed experimental design refer to (Gugger *et al.* 2015). In short, seeds were germinated in spring 2012 and seedlings were later transferred into multitrays (4 cm Ø *6*9=54 pots) filled with low-nutrient soil (Anzuchterde Ökohum, Herrenhof, Switzerland). In early July, plants were transported to the common gardens and transplanted into bigger pots (11.5*11.5*21.5 cm) with identical soil. At each site, 12 individuals per species were placed in the control beds and 12 in the beds receiving rain-shelters (i.e. drought treatment), leading to a full factorial design including 12 replicates * 28 species (14 mid and 14 high elevation species) * 2 sites (mid/high) * 2 treatments (control/dry) = 1344 individuals in total. Rain-shelters were installed after two weeks of acclimation and consisted of triangular aluminium frames with a base area of 2.4 x 3.0 m and a height of 1.2 m, covered by a UV-B transmissible greenhouse film (Luminance AF Window, Folitec, Germany; Samuel Schmid and Michael Scherer-Lorenzen, personal communication). A minimal water input was provided every two weeks during the growth period by

distributing 20 l of rainwater equally over the plants in both the control and the drought treatment.

Table 1 Overview of the congeneric pairs of mid and high elevation species included in our study (or subspecies in the case of *Anthyllis*, *Silene* and *Trifolium*). Mid elevation species ranged from 300 to 1000 m a.s.l., and high elevation species ranged from 1600 – 2400 m a.s.l. (Lauber & Wagner, 2001; Aeschiman et al., 2004). For details on the natural range of distribution of each species refer to Gugger *et al.* (2015)

Family	Mid elevation species	High elevation species
Lamiaceae	*Acinos arvensis* (Lam.) Dandy	*Acinos alpinus* (L.) Moench
Poaceae	*Anthoxanthum odoratum* L.	*Anthoxanthum alpinum* Löve
Fabaceae	*Anthyllis vulneraria ssp. vulneraria* L. s.l.	*Anthyllis vulneraria ssp. alpestris* Schult
Brassicaceae	*Arabis hirsuta* L.	*Arabis alpina* L. s.l.
Campanulaceae	*Campanula rotundifolia* L.	*Campanula scheuchzeri* Vill.
Asteraceae	*Centaurea scabiosa* L. s.l.	*Centaurea montana* L.
Caryophyllaceae	*Dianthus deltoides* L.	*Dianthus sylvestris* Wulfen
Rosaceae	*Geum urbanum* L.	*Geum montanum* L.
Fabaceae	*Lotus corniculatus* L.	*Lotus alpinus* Ramond
Fabaceae	*Onobrychis viccifolia* Scop.	*Onobrychis montana* DC.
Poaceae	*Phleum phleoides* (L.) Karsten	*Phleum alpinum* L.
Plantaginaceae	*Plantago lanceolata* L.	*Plantago alpina* L.
Caryophyllaceae	*Silene vulgaris ssp. vulgaris* (Moench) Garcke s.l.	*Silene vulgaris ssp. glareosa* (Jord.) Marsd.-Jon & Turill
Fabaceae	*Trifolium pratense ssp. pratense* L.	*Trifolium pratense ssp. nivale* (Koch)

It follows that the difference in soil water availability between the two treatments equalled the amount of natural precipitation (blocked by the rain-shelters in the drought treatment). Rain-shelters were removed after the first growing season to allow plants to overwinter under snow-cover. After snowmelt in spring 2013, rain-shelters were re-installed (mid-May at the low common garden and mid-June at the high common garden).

At each site, data loggers (TidBit v.2 UTBI-001; Onset Computer Corporation, Bourne, MA, USA) recorded temperatures at 0.5 m above the ground in both treatments (control and drought) to assess possible warming effects of rain-shelters. Similarly, light intensity loggers (Hobo pendant light data logger 64K-UA-002-64, Onset Computer Corporation, Bourne, MA, USA) were installed in each common garden and treatment to control for shading effects induced by the rain-shelters. Volumetric soil moisture content (VSCM m^3 m^{-3}) was measured monthly on a subset of pots in each treatment with a HH2 Moisture Meter and a Theta Probe type ML2x (Delta-T Devices, Cambridge, England).

Abiotic treatment effect

Over the growing season (from May to October 2013), the recorded temperature

averaged 15.7 °C at the lower site and 11.3 °C at the higher site (Table 2), and differed on average by 4.4 °C between both common gardens. Rain-shelters only marginally increased the temperature of the plant beds by 0.3 °C on average (Table 2). Light intensity (measured in klux at 1 PM, Table 2) was greater at the higher site but at both sites rain-shelters intercepted c. 30% of light without having limiting effects on plant growth (see Fig.11.11 in Körner 2003). Volumetric soil moisture content (VSMC in m^3 m^{-3}, Table 2) was significantly reduced (at least six-fold) in the drought treatment relative to the control at both common gardens (W = 900, $P = 10^{-4}$; W = 844.5, $P = 10^{-4}$, respectively).

Table 2: Mean temperature, light intensity and volumetric soil moisture content (VSMC) for each treatment averaged over the second growing season (May-October 2013).

	Temprature (°C)	Light Intensity (klux)	VSMC (m^3 m^{-3})
Low site / Control	15.5	11.53	0.4
Low site / Dry	15.9	8.45	0.06
High site / Control	11.2	13.98	0.48
High site / Dry	11.4	10.12	0.08

Assessment of plant traits and fitness proxies

At the end of the first growing season in 2012 (12 weeks after transplantation, from October 1 to 4), survival of individuals was recorded. Aboveground biomass was harvested at c. 2 cm above the ground, stored in individual parchment bags, dried for 72h at 80°C and weighed to obtain dry mass. Specific leaf area (SLA) was measured during harvest by taking circular corings from three newly grown, mature leaves per individual, while avoiding the central leaf vein (Scheepens *et al.* 2010). The diameter of the corings differed between species and ranged between 2.5 and 10 mm depending on leaf size. The three leaf corings from one individual were pooled in individual parchment bags, and dried for 48h at 60 °C. Leaf corings of one individual were weighed together to a precision of 0.0001 g. SLA was calculated for every individual by dividing the area of corings by their average dry mass (Perez-Harguindeguy *et al.* 2013).

At the beginning of the second growing season (2013), over-winter survival of individuals was recorded before re-installing the rain-shelters. Final harvest was done from September 15 to 17 at the lower common garden and from October 15 to 17 at the higher common garden. The intentional difference between both harvests allowed plants to grow for 18 weeks at both sites. For every individual, aboveground biomass was harvested at ground level, separated into vegetative and reproductive biomass and stored in parchment bags. Reproductive biomass includes flower heads and flower stems. Individual root biomass, including all belowground organs, was sampled from pots and additional roots were dug up when they had grown out of the pots (rare occurrence). After roughly cleaning roots of soil they were stored in parchment bags. All samples were kept refrigerated until transporting them back to the laboratory (max. three days), where vegetative and reproductive biomass was dried for 72h at 80 °C and weighed for dry mass. Root samples were carefully washed to remove all sediment particles above a 2 mm mesh sieve

to minimize loss of fine roots. Clean roots were dried for 72h at 80 °C and weighed. Plant mass fractions (Poorter *et al.* 2012b) were calculated as the proportion of total plant biomass allocated to each structure (RMF: root mass fraction, FMF: flower mass fraction).

Degree of phenotypic plasticity

The degree of phenotypic plasticity in response to warming and drought was estimated as a Phenotypic Plasticity Index (Pi_v) (Valladares *et al.* 2006). This index was calculated as the difference between the maximum and the minimum mean value of a given trait and species over all treatment combinations divided by the maximum mean, which serves to standardize the index ranging from zero (no plasticity) to one (maximum plasticity). The Pi_v was examined for the functional plant traits (i.e. SLA, RMF, FMF) of every species, in order to compare the degree of phenotypic plasticity between mid and high elevation species for traits related to but not directly indicative of plant fitness (i.e. biomass).

Statistical analysis

To test if the transplantation and drought treatment had an effect on plant functional traits and fitness proxies of mid and high elevation species, linear mixed-effect models were applied. 'Elevation' (mid elevation or high elevation site), 'drought' (control or drought treatment), 'origin' of species (mid elevation and high elevation species) were included as fixed effects, along with their respective two-way and three-way interactions. To account for variances between species, species nested within genus was included as random effect in the models. The environmental effects of 'elevation' and/or 'drought' indicate trait variation due to different environmental conditions (i.e phenotypic plasticity), while the 'origin' of species effect indicates differences between mid and high elevation species. The interaction between 'origin' of species and 'elevation' and/or 'drought' indicates a difference in the responses to environmental conditions between mid and high elevation species. All proportions were arc sine transformed prior to analysis (Crawley 2007). Initially, the growth form, taxonomic and functional group of species were included in the models to check for patterns induced by these factors, but these terms were removed because they were never significant. All linear mixed-effect models where performed with the 'lmerTest' package for R software (Kuznetsova *et al.* 2013) and based on Type 3 errors and Satterthwaite approximation for denominator degrees of freedom. We report *F*-values and p-values for fixed effects and χ^2-values and p-values for random effects using the "rand" function in lmerTest. Normality was verified for all variables to ensure accuracy of the estimated p-values (Pinheiro *et al.* 2000). *Post-hoc* Tukey HSD tests for multiple comparisons were performed using the 'multcomp' package (Hothorn *et al.* 2014) for R software.

The number of individuals that survived the first growing season and the following winter was counted at each site and for each treatment and analyzed using Fisher's Exact Test for Count Data.

Finally, to test for differences in the degree of phenotypic plasticity of focal plant traits between mid and high elevation species, the calculated Phenotypic Plasticity Index (Pi_v) was analysed with a paired Wilcoxon signed rank test (accounting for species genera).

All the analyses were performed on R version 3.0.2 software (R Development Core Team, 2013).

Results

Fitness proxies (survival and biomass)

96.7% of individuals survived transplantations to the common gardens and the first growing season. Not surprisingly, aboveground biomass differed between species nested within genus, because of inherent differences in productivity (Table 3; χ^2=768, P<10^{-4}). Although certain genera produced larger plants (i.e. *Anthyllis*, *Silene*) or smaller plants (i.e. *Campanula*, *Dianthus*), grouping of species in functional and taxonomic groups or growth forms did not yield further insight (factors were subsequently removed from final models). However, after the first growing season (c.12 weeks in 2012) interesting overall patterns emerged between grouped mid and high elevation species in response to elevation and manipulated water availability (Fig. 1a). Specifically, aboveground biomass decreased significantly with elevation for both mid and high elevation species (Fig. 1a; Table S1). On average, mid and high elevation species differed in their response to transplantation, as indicated by a significant interaction between elevation and origin of species (Table 3; F=28.7, P<10^{-4}). High elevation species had a consistently lower biomass than mid elevation species at both sites, but this effect was significant only at the lower elevation site (Fig. 1a). Furthermore, a significant interaction between drought and origin of species was found (Table 3; F=4.29, P=0.03). While drought generally decreased aboveground biomass for both mid and high elevation species, the negative effect of drought was significant only for mid elevation species at the lower site (Fig. 1a).

Survival assessment in 2013 revealed that c. 25% of individuals had died over winter 2012/13. Mortality was however independent of site of transplantation, treatment and origin of species (Fisher's Exact Test for Count Data: P=0.33). Additionally, another 13% of individuals were damaged by herbivores or were not reproductive during the following growing season. This resulted in highly unbalanced data across treatment combinations for species of 5 genera (*Centaurea, Geum, Onobrychis, Silene and Trifolium*), leading to the complete exclusion of these genera from analysis of data collected in 2013 to avoid any statistical biases.

After the second growing season (18 weeks), total biomass still differed between species nested within genus (Table 3; χ^2=294, P<10^{-4}). Differences between genera were larger than those between species pairs within genera, with some being inherently larger (i.e. *Anthyllis*, *Lotus*, *Silene*), compared to others (i.e. *Campanula*, *Dianthus*). More importantly, total biomass differed across treatment combinations, as revealed by a significant interaction between elevation and drought (Table 3; F=4.79, P=0.02). While drought marginally increased total biomass of mid elevation species at the lower site, total biomass of mid elevation species significantly decreased with drought at the high elevation site (Fig. 1b, Table S1). High elevation species were only marginally affected by drought at the lower site, yet total biomass of plants was significantly reduced when grown under dry conditions at the higher site relative to the control treatment at the lower site (Fig. 1b, Table S1).

Specific leaf area

Across the sites and treatments, specific leaf area ranged from 25.7 ± 0.30 $mm^2\ mg^{-1}$ at the lower elevation site, under control conditions to 15.8 ± 0.13 $mm^2\ mg^{-1}$ at the high elevation site under dry conditions (Fig. 2a, Table S1). Specific leaf area also differed between species nested within their genus

Table 3: Linear mixed effect model for the responses of functional traits to the elevation and drought treatment, the origin of the species (mid vs. high elevation species) and their respective interactions. We report *F*-values and p-values for fixed effects and χ^2-values and p-values for random effects. The significant P-values are shown in bold ($P < 0.05$).

	Above-ground biomass (g)			Total biomass (g)			SLA ($mm^2\ mg^{-1}$)			Root Mass Fraction			Flower Mass Fraction		
	df	F / χ^2	P	*df*	F / χ^2	*P*	*df*	F / χ^2	P	*df*	F / χ^2	P	*df*	F / χ^2	P
Elevation	1	254.53	**$<10^{-4}$**	1	3.07	0.08	1	331.45	**$<10^{-4}$**	1	0.002	0.95	1	0.41	0.53
Drought	1	20.95	**$<10^{-4}$**	1	14.33	**0.0002**	1	265.84	**$<10^{-4}$**	1	24.16	**$<10^{-4}$**	1	27.36	**$<10^{-4}$**
Origin	1	3.96	0.06	1	1.57	0.24	1	13.01	0.77	1	9.89	**0.01**	1	0.33	0.58
Elevation : drought	1	2.85	0.09	1	4.79	**0.02**	1	36.76	**$<10^{-4}$**	1	38.82	**$<10^{-4}$**	1	0.35	0.55
Elevation : origin	1	28.69	**$<10^{-4}$**	1	0.36	0.54	1	0.51	0.47	1	3.58	0.05	1	10.45	**0.001**
Drought : origin	1	4.29	**0.03**	1	0.03	0.85	1	6.21	**0.012**	1	1.52	0.22	1	0.05	0.81
Elevation : drought : origin	1	2.46	0.11	1	2.31	0.12	1	0.002	0.96	1	0.45	0.51	1	1.26	0.26
Species / genus	1	768.8	**$<10^{-4}$**	1	294	**$<10^{-4}$**	1	830	**$<10^{-4}$**	1	463	**$<10^{-4}$**	1	188	**$<10^{-4}$**

Table 4: Mean ± SD Phenotypic Plasticity Indices (Pi_v) for key functional traits (SLA, RMF, FMF) compared between mid- and high elevation species with a paired Wilcoxon test. We also report ranges of P_{iv} (in parentheses) for mid- and high elevation species to highlight species-specific responses.

	Pi_v mean (range) of mid elevation species	Pi_v mean (range) of high elevation species	V, P
SLA	0.41 ± 0.10 (0.24 – 0.56)	0.45± 0.11 (0.20 – 0.58)	V = 23, P = 0.13
RMF	0.29 ± 0.15 (0.09 – 0.54)	0.24 ± 0.08 (0.10 – 0.35)	V = 37, P = 0.09
FMF	0.56 ± 0.19 (0.28 – 0.89)	0.56 ± 0.24 (0.27 – 0.91)	V = 24, P = 0.9

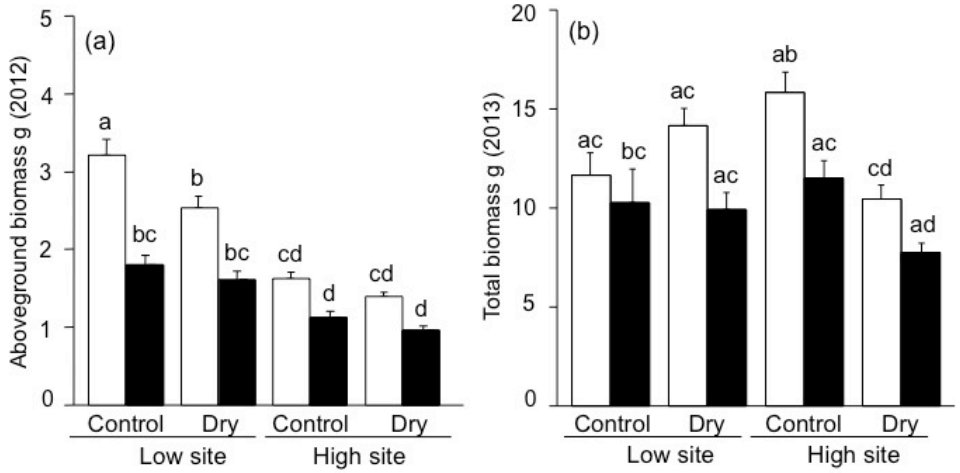

Fig. 1: Mean ± 1 SE of aboveground biomass (a) measured in 2012, and total biomass (b) measured in 2013, for mid elevation species (white bars) and high elevation species (black bars) in each treatment combination (i.e. low and high elevation, control and drought). Results from *post hoc* Tukey tests can be seen in the letter contrasts.

* Aboveground biomass was measured in 2012 on a total of 1300 individuals, while the total biomass was measured in 2013 on the surviving individuals after removal of 5 genera (n = 556

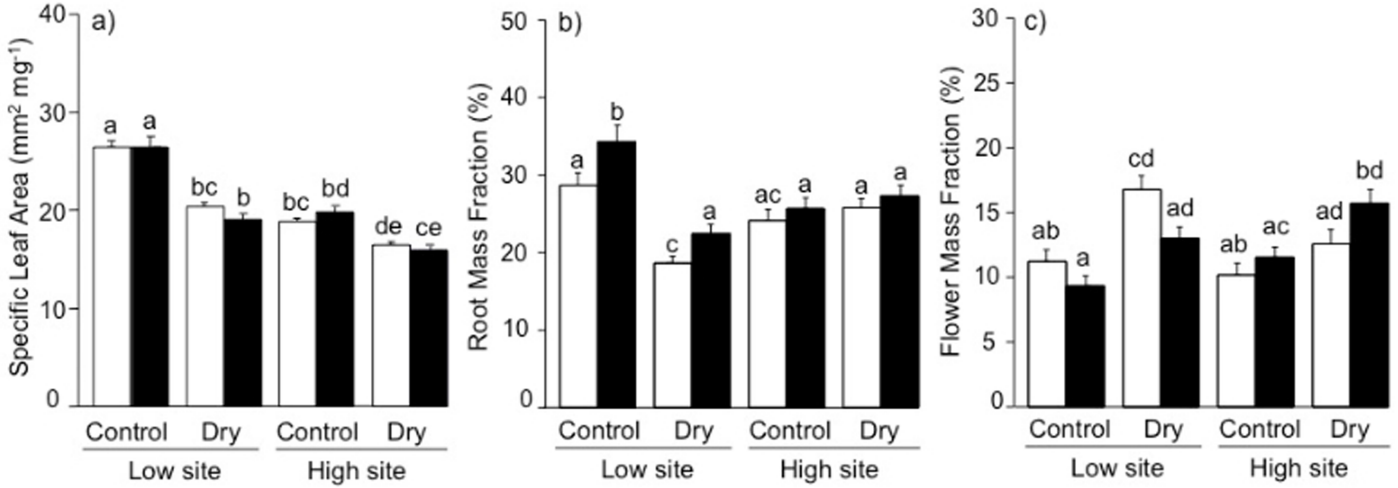

Fig. 2: Mean ± 1 SE of key functional plant traits (a) Specific Leaf Area, (b) Root Mass Fraction, and (c) Flower Mass Fraction in response to treatment combinations (i.e. low or high elevation, control and drought). Mid elevation species are represented in white bars and high elevation species in black bars. Results from *post hoc* Tukey tests can be seen in the letter contrasts.

* SLA was measured in 2012 on a total of 1300 individuals, while the RMF and the FMF were measured in 2013 on the surviving individuals after removal of 5 genera (n = 556).

(Table 3; χ^2=830, P<10^{-4}). On average, SLA decreased with elevation and drought, and the negative effect of drought was more pronounced at the lower site (Fig. 2a, Table S1), as indicated by the significant interaction between elevation and drought (Table 3; F=36.7, P<10^{-4}). Additionally, the negative effect of drought on SLA was also more pronounced for high elevation species, leading to a significant interaction between drought and origin (Table 3; F=6.2, P=0.01). Specific leaf area was however very similar between mid and high elevation species within each site (Table 3; F=13.01, P=0.7, and Tukey; Fig. 2a).

Biomass allocation (to roots and reproductive structures)

On average, plants allocated 26% of total biomass to belowground structures and 13% to reproductive structures (61% to vegetative structures). While the proportion of biomass allocated to roots or reproductive structures differed between species nested within their genus (Table 3; χ^2=463, P<10^{-4}, χ^2=188, P<10^{-4}, respectively), interesting patterns emerged when averaged across mid and high elevation species.

The proportion of total biomass allocated to belowground structures (Root Mass Fraction: RMF, Fig. 2b) differed significantly between mid and high elevation species, as indicated by a significant origin effect (Table 3; F=9.89, P=0.01). Indeed, RMF of high elevation species was significantly higher compared to mid elevation species when grown under control conditions at the lower site, and marginally higher compared to their mid elevation congeners in all other treatment combinations (Fig. 2b, Table S1). For both species' groups, RMF was surprisingly highest when grown at the lower site under control conditions, but drought had opposite effects at both sites, as revealed by the significant interaction between elevation and drought (Table 3; F=38.8, P<10^{-4}). For both mid and high elevation species, drought significantly decreased the allocation to roots at the lower site relative to the control treatment, while allocation to roots was only marginally increased at the higher site (Fig. 2b).

The investment in reproductive structures (Flower Mass Fraction: FMF, Fig. 2c) differed between elevation of transplantation and species' origin, as indicated by the significant interaction between elevation and origin (Table 3; F=10.45, P=0.001). On average, species tended to have a higher FMF when growing at their elevation of origin relative to their foreign congeners (not revealed by individual *post-hoc* test Fig. 2c). Moreover, drought had a significant effect on the FMF (Table 3; F=27.4, P<10^{-4}). Specifically, both mid and high elevation species significantly increased the allocation to reproductive structures when growing under limited water conditions at their elevation of origin (Fig. 2c, Table S1).

Phenotypic Plasticity Index (Pi_v) of mid and high elevation species

The phenotypic plasticity index did not significantly differ between mid and high elevation species for the measured plant functional traits (Table 4). The Root Mass Fraction was the only trait for which a marginally lower Pi_v was found for high elevation species (Table 4; P < 0.10). The phenotypic plasticity indices were however highly species and trait specific (see ranges Table 4). For example, plasticity in SLA ranged from 0.24 to 0.56 in mid elevation species and from 0.20 to 0.58 in high elevation species. Plasticity in RMF ranged from 0.09 to 0.54 in mid elevation species and from 0.10 to 0.35 in high elevation species. The highest ranges were found for

plasticity in FMF, which ranged from 0.28 to 0.89 in mid elevation species and from 0.27 to 0.91 in high elevation species. Finally, from the average Pi_v's and their ranges, it also becomes apparent that the FMF was the most plastic trait, SLA had an intermediate degree of plasticity and the RMF was the least plastic trait (Table 4).

Discussion

In the present study, we investigated the effects of changes in temperature (through transplantations to different elevations) and soil water availability on phenotypic variation in key functional plant traits (i.e. SLA, RMF, FMF) of 14 congeneric pairs of mid and high elevation species. We further examined if trait plasticity varied in direction and magnitude between species originating from mid and high elevation.

Plant productivity in response to elevation and drought

After the first growing season, plant productivity, measured as the aboveground biomass, was in accordance with expectations as it decreased with elevation and lower temperatures (Fig. 1a). Positive warming effects indicate that plant growth is constrained by low temperatures (Körner 2003). A moderate warming could thus have beneficial effects on high elevation plant performance and productivity, as suggested by a meta-analysis of *in situ* warming experiments with arctic and alpine tundra species (Arft *et al.* 1999) and by a climate chamber warming experiment on three grassland species (Frei *et al.* 2014b). Drought stress, however, had a negative effect on plant productivity, especially for mid elevation species and significantly reduced the production of aboveground biomass (Fig. 1a). This result confirms the efficiency of our drought treatment, which reduced the volumetric soil moisture content six-fold, and that water availability is an important limiting factor for plant productivity (Lambers *et al.* 1998).

Variation in total biomass in response to the different treatment combinations during the second growing season was less consistent (Fig. 1b). Although, total biomass was significantly reduced by drought at the high site, at the lower site drought had no effect on the total biomass of high elevation species and seemed to marginally increase the productivity of mid elevation species. Increased biomass productivity of grasslands subjected to drought stress has also been reported by Gilgen *et al.* (2009) and was explained by improved soil oxygenation. Higher soil oxygen concentrations are expected to increase soil mineralisation rates and consequently nutrient availability, which could rapidly lead to higher plant productivity (Gilgen *et al.* 2009, Brilli *et al.* 2011).

Finally, while inherent differences in productivity were detected between genera, no significant effects of growth forms or taxonomic and functional groups were detected. However, across the species' origin we detected that on average high elevation species always had lower aboveground and total biomass than mid elevation species. This result highlights the fundamental differences in growth strategies between species from mid and high elevation, with high elevation species displaying smaller or even dwarfed morphologies (Billings *et al.* 1968, Körner *et al.* 1987, Körner 2003), allowing them to better withstand harsh alpine conditions (i.e. temperature extremes, snow, wind, irradiance etc.). Clearly, our results confirm that these differences in growth form are genetically determined.

Plastic responses of key functional traits to elevation and drought treatment

Specific leaf area showed substantial phenotypic plasticity after 12 weeks, as indicated by a significant decrease in SLA with increasing elevation and drought (Fig. 2a), in accordance with literature (Prock *et al.* 1996, Scheepens *et al.* 2010, Pang *et al.* 2011, Poorter *et al.* 2012a). At both sites, drought stress reduced SLA and the highest SLA values were found for leaves of individuals grown under control conditions at the mid elevation site and lowest values were found under dry conditions at the high elevation site. However, SLA values did not vary between the dry treatment at mid elevation and the control treatment at high elevation, possibly implying that transplantations to the higher site and the drought treatment at low elevation exerted comparable pressures on plants, resulting in similar SLA values. Additionally, SLA values of mid and high elevation species did not differ within treatment combinations, suggesting similar responses to external conditions. The decrease in SLA with increasing elevation and drought stress can be achieved through increases in leaf density and/or leaf thickness (Körner 2003, Poorter *et al.* 2009). Though we did not measure these traits separately, Scheepens *et al.* (2010) found in *Campanula thyrsoides* that leaf thickness significantly decreased with elevation and thus explained the decrease in SLA through substantial increases in leaf density, leading to smaller cells and more cells per unit leaf volume. This might also be true in our case, especially in the event of drought stress, which restricts cell expansion by decreasing internal turgor pressure of the cells (Sharp *et al.* 1989, Tardieu *et al.* 2000). Overall, high plasticity in SLA is highly advantageous as it allows plants to adjust growth rate, leaf longevity and stress tolerance to prevailing environmental conditions (Wright *et al.* 2004, Scheepens *et al.* 2010).

The proportion of total biomass allocated to belowground structures, measured as the root mass fraction (RMF), only decreased under drought stress at the mid elevation site, and was unaffected by site elevation in general. This result is counter to predictions, as investment in roots usually increases with elevation (Körner 2003) and under limited soil water availability (Bell *et al.* 1999, Heschel *et al.* 2004, Larcher *et al.* 2010). Similarly to our results, Kreyling *et al.* (2008), Gilgen *et al.* (2009), and Backhaus *et al.* (2014) found small increases or no alterations in plant belowground biomass in response to limited soil water availability. While we cannot exclude that some root material was lost during sampling or cleaning, leading to biases in our data, we rather hypothesize that the similar values in RMF are due to the fact that root morphology differed between treatment combinations. Körner *et al.* (1987) showed that with increasing elevation, investment in fine roots increases, and a similar result was found in response to low water potential (Fraser *et al.* 1990). Fine roots, which have a thinner diameter and are less lignified and suberised than coarse roots (Lavelle *et al.* 2005), probably result in less dry weight than thicker roots and we argue that this morphological difference could potentially explain that changes in RMF between site elevations and soil water availability were relatively small. In accordance with literature, our results however showed that high elevation species generally invested more biomass in belowground structures relative to their mid elevation congeners (Billings *et al.* 1968, Körner 2003). Additionally, among the studied functional traits, RMF was the least plastic trait (Pi_v c.

0.265) and showed particularly little variation in high relative to mid elevation species, and lesser variation when mid elevation species were grown at the higher site, indicating the constraints acting on allocation patterns at high elevation.

The proportion of total biomass allocated to reproductive structures, measured as the flower mass fraction (FMF) tended to be greater for mid elevation species when growing at the lower elevation site and for high elevation species when growing at the high elevation site. As the FMF is closely associated with seed production and plant fitness, these results seem to indicate a home-site advantage of species to the conditions at their habitat of origin (Joshi *et al.* 2001, Blanquart *et al.* 2013). Furthermore, drought also increased the allocation to reproductive structures for mid and high elevation species at their respective elevation of origin. These results suggest a prioritisation of reproduction at the expense of growth under drought stress. Interestingly, FMF showed the highest plasticity among the studied plant traits (Pi_v of 0.56), probably indicating the importance of adjusting this trait to environmental conditions to maintain fitness homeostasis.

Degree of phenotypic plasticity compared between mid and high elevation species

We found very little evidence for differences in the degree of phenotypic plasticity in key plant traits between mid and high elevation species. Only the plasticity in RMF was marginally smaller for high elevation species (Table 4). This indicates that high elevation species were less capable of adjusting the allocation to belowground structures to changing external conditions, probably reflecting their genetically fixed higher allocation to below-ground structures. Similar results were found by Frei *et al.* (2014b), where plasticity was reduced in only a single trait (leaf length) in high elevation populations of *Trifolium montanum*. Consequently, the magnitude but also the direction of plasticity in key plant traits in response to transplantations and soil water availability in mid and high elevation species was similar, suggesting rather uniform responses to climate change between these two groups of species (Frei *et al.* 2014b).

More generally, and contrary to our hypothesis, phenotypic plasticity did not seem to depend on environmental heterogeneity more commonly observed at high elevation (Scherrer *et al.* 2011). In contrast to the general consensus that phenotypic plasticity should be selected for in heterogeneous environments (Via *et al.* 1985), other studies also reported no differences in plant trait plasticity compared between populations from habitats with constant and more variable environmental conditions (Heschel *et al.* 2004, Franks 2011). These previous results, in combination with our study, indicate that increased environmental variation does not necessarily lead to a greater degree of functional plasticity. Two combined factors are predicted to favour selection for phenotypic plasticity: when the rates of environmental change are similar or slower than the response rate of an organism and when said change is highly but not completely predictable (Scheiner 1993). In alpine environments, change might be rather unpredictable and could thus explain why the advantages of being plastic in response to environmental heterogeneity do not necessarily outweigh the costs (i.e. maintenance, production and information acquisition cost; DeWitt *et al.* 1998).

Although no difference was found in functional plasticity between mid and high elevation species, some traits were more

plastic than others. Specific leaf area and the allocation to reproductive structures (FMF) were highly plastic in response to treatment combinations, while the allocation to belowground structures (RMF) was comparatively less plastic, hence more strongly genetically controlled. This result indicates constrained phenotypic plasticity in this specific trait, which could be related to potential stabilizing selection acting on allocation patterns at high elevation. Constrained plasticity was also found in the reproductive phenology of high elevation species, which was monitored in a parallel study during the second year of this experiment (Gugger *et al.* 2015). Particularly, high elevation species were less plastic than their lower elevation congeners in the timing of peak flowering, suggesting that adaptation to short growing seasons in alpine environments limits the potential for plasticity of flowering phenology in high elevation species in response to environmental change (Gugger *et al.* 2015), and leads to a higher genetic canalization of the timing of peak flowering (Price *et al.* 2003, Pigliucci *et al.* 2006, Ghalambor *et al.* 2007). This however does not seem to apply to all functional traits of high elevation species, as we have shown here that SLA and FMF were highly and equally plastic in mid and high elevation species.

Conclusion

To conclude, both mid and high elevation species displayed great functional plasticity in key plant traits related to ecophysiological characteristics in response to changing temperatures and soil water availability. As the direction and magnitude of functional plasticity was similar between mid and high elevation species, our results suggest rather uniform responses of these species groups to climate change. While plasticity in functional traits was highly species and trait specific, the general capacity of species to respond plastically to environmental changes may offer a short-term strategy to face climate change.

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Supplementary data

Table S1: Mean ± SE of aboveground biomass, total biomass, SLA, RMF and FMF reported for the species groups (mid elevation and high elevation species) and treatment combinations: A (low site/control), B (low site/dry), C (high site/control), D (high site/dry).

		Treatment combinations			
	Species group	A	B	C	D
Aboveground biomass (g)	Mid elevation species	2.99 ± 0.07	2.42 ± 0.05	1.53 ± 0.03	1.32 ± 0.02
	High elevation species	1.71 ± 0.04	1.51 ± 0.04	1.07 ± 0.03	0.91 ± 0.02
Total biomass (g)	Mid elevation species	11.65 ± 0.50	14.15 ± 0.38	15.82 ± 0.45	10.45 ± 0.31
	High elevation species	10.26 ± 0.74	9.91 ± 0.38	11.51 ± 0.38	7.75 ± 0.21
Specific leaf area ($mm^2.mm^{-1}$)	Mid elevation species	25.59 ± 0.23	20.19 ± 0.13	18.73 ± 0.12	16.55 ± 0.12
	High elevation species	25.74 ± 0.35	18.67 ± 0.17	19.47 ± 0.21	15.86 ± 0.14
Root mass fraction	Mid elevation species	0.28 ± 0.007	0.19 ± 0.004	0.24 ± 0.006	0.26 ± 0.005
	High elevation species	0.34 ± 0.009	0.23 ± 0.005	0.26 ± 0.006	0.27 ± 0.006
Flower mass fraction	Mid elevation species	0.11 ± 0.004	0.17 ± 0.004	0.10 ± 0.004	0.13 ± 0.005
	High elevation species	0.09 ± 0.003	0.13 ± 0.003	0.11 ± 0.003	0.16 ± 0.004

Chapter 4

Past selection explains differentiation in flowering phenology of nearby populations of a common alpine plant

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Past selection explains differentiation in flowering phenology of nearby populations of a common alpine plant.

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Abstract

• The timing of and relative investment in reproductive events are crucial fitness determinants for alpine plants, which have limited opportunities for reproduction in the cold and short growing seasons at high elevations. We use the alpine *Anthyllis vulneraria* to study whether flowering phenology and reproductive allocation have been under diversifying selection, and to assess genetic diversity and plastic responses to drought in these traits.

• Open-pollinated maternal families from three populations in each of two regions from the Swiss Alps with contrasting precipitation were grown in low and high soil moisture in a common garden. We measured onset, peak, and end of flowering, as well as vegetative and reproductive aboveground biomass. Population differentiation for each character (Q_{ST}) was compared to differentiation at neutral microsatellite loci (F_{ST}) to test for past selection.

• We found population differentiation in onset and peak of flowering which results from natural selection according to Q_{ST}-F_{ST}. End of flowering and biomass were not significantly differentiated among populations. Reduced soil moisture had no consistent effect on mean onset of flowering, and advanced peak and end of flowering by less than one week. Reproductive biomass was strongly decreased by lowered soil moisture. No genetic variation within or among populations was found for plasticity in any trait measured.

• The results suggest past heterogeneous selection on onset and peak of flowering in alpine *Anthyllis vulneraria* and potentially indicate local adaptation to differences in snowmelt date over distances < 5 km. Limited variation in plastic responses to reduced soil moisture suggests that soil moisture might not vary between populations.

Keywords: Local adaptation; Q_{ST}-F_{ST} comparison; phenotypic plasticity; drought

Introduction

Evolution by means of divergent natural selection in spatially heterogeneous environments is considered the major cause of phenotypic variation (Linhart and Grant 1996; Schluter 2009). Apart from divergent natural selection, phenotypic variation among populations can also result from genetic drift – random variation in allele frequencies eventually resulting in the fixation of alleles in some populations and their extinction in other populations (Wright 1931). Furthermore, population differentiation may result from the expression of different phenotypes by the same genotypes in different environments, a phenomenon referred to as phenotypic plasticity (Bradshaw 1965). In alpine plants, both local adaptation and phenotypic plasticity have been hypothesized particularly important due to the steep environmental gradients across short geographical distances. On the other hand, populations of alpine plants are frequently small owing to the highly structured landscape, thereby intensifying genetic drift. The relative significance of genetic drift, local adaptation, and plasticity for population differentiation in alpine environments, however, is a question that remains insufficiently answered (Leimu and Fischer 2008; Frei et al. 2014).

The timing of reproductive events and the allocation of resources to reproduction are particularly crucial fitness determinants in the highly seasonal alpine environments (Rathcke and Lacey 1985; Ozenda 1995; Körner 2003). As temperature decreases along rising elevation with 0.55 °C/100 m, the snow free period and the time window for reproduction narrow down (Schroeter 1923). At high elevations, flowering phenology is therefore tightly linked to the date of snowmelt (Hülber et al. 2006). Among animal-pollinated plant species, the phenology and allocation of reproductive effort must also be coordinated with pollinator abundances and behaviour (Müller 1881; Kudo 1996). Temperature and the date of snowmelt at high elevations are subject to strong microhabitat effects, which may outweigh elevational effects of a few hundred meters (Scherrer and Körner 2010; Wheeler et al. 2014). Therefore the tight links of temperature and snowmelt with phenology and pollinator behaviour (Bergman et al. 1996) is expected to result in strong population differentiation in flowering phenology. The sensitivity of flowering phenology to external conditions can further facilitate reproductive isolation via asynchronous flowering time and therefore promote differentiation and local adaptation (Linhart and Grant 1996; Hall and Willis 2006; Hülber et al. 2010).

Contrarily, the evolution of phenotypic plasticity is expected when genotypes or lineages are likely to experience various external conditions due to high spatial or temporal environmental heterogeneity (Sultan and Spencer 2002). Flowering phenology is an inherently plastic trait that is strongly environmentally controlled through temperature, photoperiod, or both (Keller and Körner 2003). Phenotypic plasticity can slow the response to selection when genetic variation in plasticity exists in a population, i.e. when all genotypes in the population do not respond to environmental change in the same way. Natural selection then cannot operate on trait means as efficiently as when environments and phenotypes are stable, because the same genotype does not have highest fitness under all conditions (Via and Lande 1985). The question, whether mean flowering time is subject to divergent selection among populations in the highly

variable alpine landscape remains rarely addressed (Scheepens et al. 2011; Scheepens and Stöcklin 2013; Frei et al. 2014).

Besides temperature and photoperiod, reproductive characters such as reproductive allocation are likely to respond to soil water availability (Caruso 2006, and references therein), because flowering incurs substantial water costs to the plant. The transpirational water loss of flowers can exceed that of leaves (Galen et al. 1999; Lambrecht 2013). Alpine plants often have big flowers relative to the vegetative body (Körner 2003). Disproportionately large flowers therefore might further raise water costs. Under drought, plants produce smaller flowers and smaller reproductive structures in general (Mal and Lovett-Doust 2005; Caruso 2006), and were also found to advance flowering phenology as a plastic as well as an evolutionary response (Dunne et al. 2003; Franks 2011). Precipitation is very variable across the European Alps as a result of the interplay of climatic patterns with the obstructing effect of mountain ranges. This leads to regions of particularly low precipitation in the deep valleys inside the highest mountain ranges (Ozenda 1985).

Here we use comparisons of quantitative trait differentiation and genetic differentiation at neutral marker loci (Q_{ST}-F_{ST} comparisons; Spitze 1993) to test for the role of past selection in shaping patterns of population differentiation in reproductive allocation and phenology in a common alpine herb. Q_{ST}-F_{ST} comparisons allow to infer natural selection as opposed to random processes such as genetic drift as a cause of population differentiation when Q_{ST} is either significantly smaller or larger than F_{ST}. Q_{ST} values smaller than F_{ST} values indicate stabilizing selection across environments, whereas Q_{ST}'s larger than F_{ST} indicate population divergence as a result of heterogeneous selection across environments. If $Q_{ST} = F_{ST}$, we have no reason to infer a role of selection as drift alone can explain the observed population differentiation. We also assess plastic responses to drought in reproductive allocation and phenology by subjecting plants to two soil moisture treatments in a common garden. We asked (i) if population differentiation in reproductive allocation and flowering phenology is likely the result of past selection and therefore adaptive evolution, and (ii) if soil moisture availability has an effect on reproductive allocation and phenology (i.e. presence of phenotypic plasticity).

Methods

Study species

Anthyllis vulneraria L. sensu lato (s.l.) is a polymorphic fabacean taxon with unclear infraspecific classification (Nanni et al. 2004; Köster et al. 2008), and consists of a self-compatible clade of short-lived herbaceous plant species very common throughout Europe. It grows preferably on calcareous meadows and scree grounds from sea level to the alpine belt up to around 3000 m a.s.l. (Hegi 1975). Here we examined three alpine populations of *Anthyllis vulneraria* in each of two regions in the Swiss Alps. Plants grow to a height of around 15-45 cm. A variable number of shoots sprout from the basal leaf rosette, each bearing 2-6 inflorescences. Each inflorescence comprises a number of 7-19 mm long white to yellow, sometimes claret to red flowers arranged in a capitulum (Hegi 1975; Navarro 1999). Shoots are usually unbranched, but may have up to three side-branches originating from the axils of evenly pinnate compound leafs. Leafs of the basal rosette consist of the enlarged terminal leaflet of a compound leaf. *Anthyllis vulneraria* is representative of a type of fabacean flower

characterized by a *pump mechanism* adapted to insect-mediated pollination (Müller 1881). Flower development of *Anthyllis vulneraria* takes approximately 4 weeks. Flowers ripen from bottom to top along a shoot and from top to bottom within a capitulum. Asynchronous flower ripening allows for geitonogamous selfing across capitulae, but suggests multiple paternity per maternal offspring. Microsatellite analyses found a variable degree of inbreeding in the studied populations (F_{IS} 0 - 0.42, unpublished results), suggesting regular outcrossing. A single flower is open and accessible to pollinators for about 6 to 7 days and produces a single seed.

Experimental procedures

In August 2012, seeds from open-pollinated wild flowers in three populations from each of two regions (eastern and western Swiss Alps near Davos and Zermatt, respectively) were sampled (Table 1; Online Resource 1). The offspring of the same maternal plant are referred to throughout the article as *seed family*. Members of a seed family presumably are mostly half-sibs, as populations are outcrossed, and the asynchronous ripening of flowers within a capitulum makes it unlikely that they are sired by the same father (Pannell and Labouche 2013). Populations are situated between 2000 m a.s.l. and 2650 m a.s.l. Distances between populations within regions range from 2 km to 18 km, and regions are 180 km apart. Regions were specifically chosen for their difference in growing season precipitation, with the Davos region getting approximately 50% more precipitation in the months of June through September than the Zermatt region (Table 1; Zimmermann and Kienast 1999). We have identified populations as belonging to *Anthyllis vulneraria* ssp. *alpestris* after Hegi (1975). Seeds were stored in the refrigerator until they were scarified and sown in early August 2013 directly into their final high mineral potting soil mixture (210 l Ökohum Anzuchterde® with 14 l sand and 8 kg pumice). 5 individuals per seed family, and 6 seed families per population were used (180 individuals in total). Seedlings were kept in the greenhouse in 10 by 10 cm pots and watered *ad libitum*. Plants were randomized twice per week. Greenhouse heating and cooling systems were set so that temperatures would not fall below 16 °C and 8 °C at day and night, respectively, nor exceed 20 °C and 10 °C at day and night, respectively. Early leaf size was measured on every individual as length*width/2 of the first true leaf as soon as it was fully developed (2 leaf stage). After 4.5 weeks, on 11-Sep-2013, seedlings were potted into larger 2 l pots into the same soil mixture and watered to carrying capacity. At the same time, plants were moved to the outside garden under a UV-B transmissible rain shelter (folitec Agrarfolien-Vertriebs GmbH, Westerburg, Germany) and arranged in a regular array, alternating between individuals of all levels of hierarchy from seed family to region. Eleven days later, on the 22-Sep-2013, treatment began by watering plants designated for the wet treatment. Alternating between seed families, 3 or 2 of the 5 individuals per seed family were allocated to the dry treatment. Subsequently, volumetric soil moisture content was monitored with a moisture meter calibrated to the soil mixture used in the experiment and plants were watered accordingly (HH2 Moisture Meter with Theta Probe ML2x, Delta-T Devices Ltd. Cambridge, England). Wet plants were watered when mean soil moisture fell below 18 %, and dry plants when soil moisture fell below 5 %. Ten different plants of each treatment were randomly chosen each time at

irregular intervals for soil moisture measurements (Online Resource 2). Plants were sitting on a thick sand bed and marginally striked roots into the sand 2cm deep at maximum. Throughout the duration of the experiment, air temperature was logged hourly with a TidbiT® v2 Temperature Logger (Onset Computer Corporation, Bourne, Massachusetts, U.S.A.; Online Resource 3). The logger was hung-up under a reversed 2 l plastic flowerpot painted in white and with perforation to allow air circulation. All plants were preventively treated with a ready-to-use fungicide powder (Maag Pirox®, Syngenta Agro AG, Dielsdorf, Switzerland) on a few occasions during growth phase, because alpine *Anthyllis vulneraria* is susceptible to mildew when grown at low elevations. *Anthyllis vulneraria* needs vernalisation to induce flowering (Halil Kesselring, personal observation), so we left plants outside over winter. During winter, from 19-Nov-2013 onwards, the rain shelter was temporarily removed and water treatment was suspended. Treatment was re-established on the 19-Mar-2014 by watering with 100ml and 60ml for wet and dry plants, respectively, and subsequently continued as described above.

Once reproductive shoots became visible, plants were checked daily and the date of the following critical stages of flowering phenology were noted for each individual: i) *onset of flowering* defined as the date when the first flower opened on an individual; ii) *peak of flowering* defined as the date at which the maximum number of open flowers was observed; iii) *end of flowering* defined as the date when the last flower opened and no more flower buds were visible. Flower opening is very easily observed in *Anthyllis vulneraria* when the brightly coloured corolla appears from the calyx, a process that takes less than 24 hours. Onset of flowering was always a representative measure because the opening of the first flower was never an isolated event, but led to the onset of flowering of the whole plant.

Once a plant had reached the flower end, aboveground biomass was harvested and dried at 75 °C for 72 hours. Aboveground biomass was then separated into the vegetative leaf rosette and into reproductive parts, and weighed to the nearest mg. We also estimated *reproductive allocation* as the ratio of reproductive biomass over total aboveground biomass.

Table 1 Coordinates (Swiss coordinate system LV03), elevation, and mean monthly precipitation during the growing season (June – September) of the six populations of *Anthyllis vulneraria* studied in the common garden. Precipitation data is interpolated from monthly precipitation data using a digital elevation model (Zimmermann and Kienast 1999).

Region	Population	Coordinates (°E/°N)	Elevation (m a.s.l.)	Summer precipitation (mm)
Davos	Schiahorn	780513.385/187874.756	2650	1463
	Casanna	782301.543/192247.969	2320	1454
	Monstein	779685.630/173389.160	2010	1225
Zermatt	Findelwald	626828.986/95475.764	2170	809
	Findelgletscher	629173.611/95175.270	2490	939
	Stafelalp	619094.320/94427.436	2280	898

Statistical analyses

We performed separate linear mixed-effects models for each of our flowering phenology variables and for the biomass variables in R version 3.0.2 (R Development Core Team 2008). In these models, *water treatment* and *region* as well as their interaction were included as fixed effects, and *population* and *seed family* and their interactions with *water treatment* were included as random terms. Each seed family was given a unique identifier, which leads to the models implicitly nesting *seed family* in *population*. Likewise, *population* was nested in *region*. In these models, a significant *water treatment* effect indicates that soil moisture availability has an effect on either flowering phenology or aboveground biomass allocation, i.e. the focal trait is plastic in response to soil moisture. A significant interaction between *water treatment* and *region* indicates that populations from both regions differ in their plastic responses to soil water availability. Analogously, a significant *seed family* effect indicates that related individuals are more similar to each other in the focal trait expression than randomly grouped individuals, and an interaction of *seed family* with *water treatment* indicates genetic variation in phenotypic plasticity among seed families within populations. In order to control for maternal effects to the maximum possible extent, early leaf size (length*width/2) was included in all models as covariate. Statistical models were computed with the lmerTest package (Kuznetsova 2013). lmerTest applies F-tests to lmer objects of the lme4 package for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and comparisons. We used type 3 errors and Satterthwaite approximations for denominator degrees of freedom. We report *P*-values, mean squares, and chi-square values that correspond to those from the model comparisons using the step function in lmerTest (i.e. likelihood-ratio tests). All random terms were specified as simple scalar terms. Phenological variables were analysed as date objects. Contrasts for fixed effects were tested using differences of least squares means as implemented in the step function of lmerTest.

Molecular analyses

20 individuals per population were scored for amplified fragments at 9 microsatellite loci. We used Spreadex® gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) to separate PCR amplicons with size differences as small as 2bp. Gels were stained with ethidium-bromide and scored by hand comparing against the M3 ladder from ELCHROM. Polymorphic microsatellites were developed to be suitable in length for analysis on Spreadex® gels (Kesselring et al. 2013). PCR programs were run in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). 35 cycles with denaturation for 30 s at 95°C, start PCR for 30 s at 95°C, locus-specific annealing temperature (50 or 52°C) for 45 s, followed by 45 s at 72°C were repeated. Termination was set to 72°C for 8 min. A detailed description of the microsatellite development and loci description can be found in Kesselring et al. (2013). The free software FreeNA (Chapuis and Estoup 2007) was used to check for null alleles. Null alleles were suggested for several loci, but taking their frequencies into account resulted in nearly identical F_{ST} estimates for each locus except locus 8. Mean F_{ST} was slightly lower with null alleles taken into account, therefore inclusion of null alleles would render tests of $Q_{ST} > F_{ST}$ less conservative. Since a low degree of

inbreeding is suggested by the data and the floral biology of *Anthyllis vulneraria*, and since blank lanes (homozygote null alleles) were only present at locus 8, we are confident that increased homozygosity at all but one locus is not due to null alleles, but results from bi-parental inbreeding and selfing. Estimation of null alleles rests on untested assumptions (e.g. a single null allele is present) and is not free of bias (Chapuis and Estoup 2007; David et al. 2007). Consequently, we preferred to remove the outlier locus with clear signals of null alleles instead of including null allele frequencies for final analyses. Genotyping error was estimated at 2.5 % (Kesselring et al. 2013). Population pairwise F_{ST}-values were calculated in GenAlEx (Peakall and Smouse 2006) based on allele frequencies. Probabilities of finding the observed F_{ST}-values are based on comparison of the observed value against 999 random permutations of the samples.

Q_{ST}-F_{ST} comparison

Q_{ST}-F_{ST} comparisons were performed for all traits to test whether population differentiation in quantitative traits is the result of natural selection. We followed the method described by Whitlock and Guillaume (2009), which provides a powerful significance test of the hypothesis that Q_{ST} is not equal to F_{ST}. For each trait, a null-distribution of Q_{ST}-F_{ST} is first constructed based on the observed within-population genetic variance for the focal trait and observed F_{ST}. Since it is based on the F_{ST}, this null-distribution is the expected distribution of Q_{ST}-F_{ST} under neutral evolution of the trait. The tail probability of the observed Q_{ST}-F_{ST} under the assumption of neutral evolution is then calculated from the null-distribution.

The software Nemo version 2.2.0 (Guillaume and Rougemont 2006) was used to calculate Weir and Cockerham's coefficients a,b, and c for each of the 8 microsatellite loci as a basis to estimate Wright's F_{ST} (Weir and Cockerham 1984). Whitlock and Guillaume (2009) provide an R script for a nonparametric bootstrap of F_{ST} values, which was used to generate 10^3 bootstrap replicates of F_{ST}, from which a probability distribution of F_{ST} was constructed. The script calculates F_{ST}'s by randomly sampling with replacement from the Weir and Cockerham coefficients calculated by Nemo a number of times equivalent to the number of loci used in the analyses. The Q_{ST} replicates were calculated by parametric bootstrapping using the Lewontin-Krakauer distribution and the observed within-population variances and observed F_{ST} according to Whitlock and Guillaume (2009). For the calculation of Q_{ST}'s, we used Spitze's (1993) formula, estimating within-population variances as 4 times the seed family variance components, and among-population variance as the population variance components from the statistical models. Some degree of inbreeding is indicated in 5 of the studied populations by heterozygote deficiencies. Therefore the assumption of half-sibs may not always hold, and render tests of $Q_{ST}>F_{ST}$ too conservative and those of $Q_{ST}<F_{ST}$ too relaxed. We have therefore repeated the analyses under the assumption of full-sibs which is the opposite extreme. Results were identical, except that significant results were even more strongly significant under the assumption of full-sibs. We only report results assuming half-sibs as this is the more realistic and more conservative assumption. We used the minimal adequate models resulting from stepwise model reduction as implemented in the step function of lmerTest for estimation of all variance components used in the Q_{ST}-

F_{ST} comparisons. The entire sample across both treatments was used for analyses to achieve reasonable sample sizes. If the minimal model did not include *population* or *seed family* those terms were re-included as they are necessary for calculations of Q_{ST}.

Results

In the experimental garden, global mean peak flowering was on the 25-April-2014. Peak flowering in the natural stands of these populations is roughly in the last week of June (Halil Kesselring, personal observation). Therefore, plants in this experiment flowered approximately 2 months earlier than the natural stands, equalling a 1h 45 min shorter photoperiod. All populations were significantly differentiated at microsatellite loci from one another (average F_{ST} = 0.079, 95% CI: [0.063, 0.098]) except populations Stafelalp and Findelwald (Table 2). Mean pairwise population differentiation within regions (F_{ST}=0.04) was lower than mean pairwise population differentiation across regions (F_{ST}=0.08, Table 2).

Effects of origin on allocation of biomass and phenology

Differentiation at the regional level was only indicated for vegetative biomass and reproductive allocation (ratio of reproductive biomass over total aboveground biomass), but not for reproductive biomass by statistical analyses (Table 3). Populations nested within region were not significantly differentiated for biomass traits (Table 3), with observed Q_{ST}'s of 0.074 and 0.11 for vegetative and reproductive biomass, respectively. Accordingly, observed Q_{ST}-F_{ST} values fell within the 95%-confidence limit of the corresponding null-distributions for both vegetative (*P*=0.666) and reproductive biomass (*P*=0.429), giving no reason to infer selection as a driver of population evolution. Variance components analysis of the random terms revealed that large amounts of variability in vegetative and reproductive aboveground biomass were explained by *seed family* (31% and 22%, respectively; Table 3), indicating high within-population genetic diversity.

The two regions were significantly differentiated for all stages of reproductive phenology (Table 3). All populations from Zermatt flowered later than any of the populations from Davos, and the difference in peak flowering date between the first population from Davos and the last one from Zermatt was more than 5 weeks (Fig. 1). Significant population differentiation within regions was indicated by the statistical models for onset and peak of flowering, but not for end of flowering (Fig. 1; Table 3). Observed Q_{ST}'s ranged from 0.020 for end of flowering to 0.353 for peak flowering (Fig. 2). The observed Q_{ST}-F_{ST} value for end of flowering fell within the 95%-confidence limit of the corresponding null-distribution (*P*=0.984), and therefore differentiation in this trait can be explained by drift alone. Q_{ST}-F_{ST} values for onset and peak of flowering were greater than expected under the null-hypothesis and appeared in the tail of the corresponding null-distributions with an associated probability of finding the observed value or one that is greater of 0.002 and 0.001, respectively. Therefore, divergent selection is indicated for onset and peak of flowering. The observed F_{ST} as inferred from the microsatellite data was 0.079 (95% CI: [0.063, 0.098]) averaged over all loci. Genetic variation within populations was indicated for all phenological variables by significant *seed family* terms (Table 3). *Seed family* explained 10%, 11%, and 37%, of the variability in the random terms for onset, peak, and end of flowering, respectively.

Table 2: Population pairwise F_{ST} values (below diagonal) with tail probabilities based on 999 random permutations of samples as implemented in GenAlEx given (above diagonal).

	Schiahorn	Casanna	Monstein	Findelgletscher	Findelwald	Stafelalp
Davos						
Schiahorn	-	0.001	0.001	0.001	0.001	0.001
Casanna	0.051	-	0.037	0.001	0.001	0.001
Monstein	0.084	0.014	-	0.001	0.001	0.001
Zermatt						
Findelgletscher	0.127	0.078	0.096	-	0.014	0.001
Findelwald	0.095	0.038	0.064	0.020	-	0.450
Stafelalp	0.098	0.039	0.085	0.052	0.000	-

Table 3 The effects of population origin, soil moisture treatment, and family membership on aboveground biomass and reproductive phenology (linear mixed-effects analyses). Early leaf size (measured as length*width/2 of the first fully developed true leaf) was included as a covariate to control for maternal effects. *** $P<0.001$, ** $P<0.01$, * $P<0.05$, *ns* not significant. F-ratios are given for fixed effects and $\chi 2$-values for random effects. Reproductive allocation is the ratio of reproductive biomass over total aboveground biomass.

	Vegetative biomass			**Reproductive biomass**			**Reproductive allocation**			**Onset of flowering**			**Peak of flowering**			**End of flowering**		
factor	df	F/$\chi 2$	*P*	df	F/$\chi 2$	*P*	df	F/$\chi 2$	*P*	df	F/$\chi 2$	*P*	df	F/$\chi 2$	*P*	df	F/$\chi 2$	*P*
early leaf size	1	15.40	**		23.35	**	1	10.33	*	1	0.02	*ns*	1	1.29	*ns*	1	0.71	*ns*
Region	1	15.77	**	1	26.17	*ns*	1	12.30	*	1	8.40	**	1	8.54	*	1	7.79	**
Treatment	1	5.40	*	1	27.06	***	1	31.26	***	1	0.09	*ns*	1	4.36	*	1	12.20	**
Region x treatment	1	4.74	*	1	3.34	*	1	8.44	*	1	0.24	*ns*	1	0.03	*ns*	1	0.27	*ns*
Population (region)	na	1.94	*ns*	na	1.60	*ns*	na	0	*ns*	na	17.51	**	na	21.37	***	na	0.88	*ns*
Seed family (population)	na	32.91	***	na	24.98	***	na	14.54	***	na	8.58	**	na	12.35	**	na	23.18	**
Population x treatment	na	0	*ns*	na	0.03	*ns*	na	0	*ns*	na	0	*ns*	na	0.29	*ns*	na	0	*ns*
Seed family x treatment	na	0	*ns*	na	1.14	*ns*	na	0	*ns*	na	0	*ns*	na	0	*ns*	na	0	*ns*

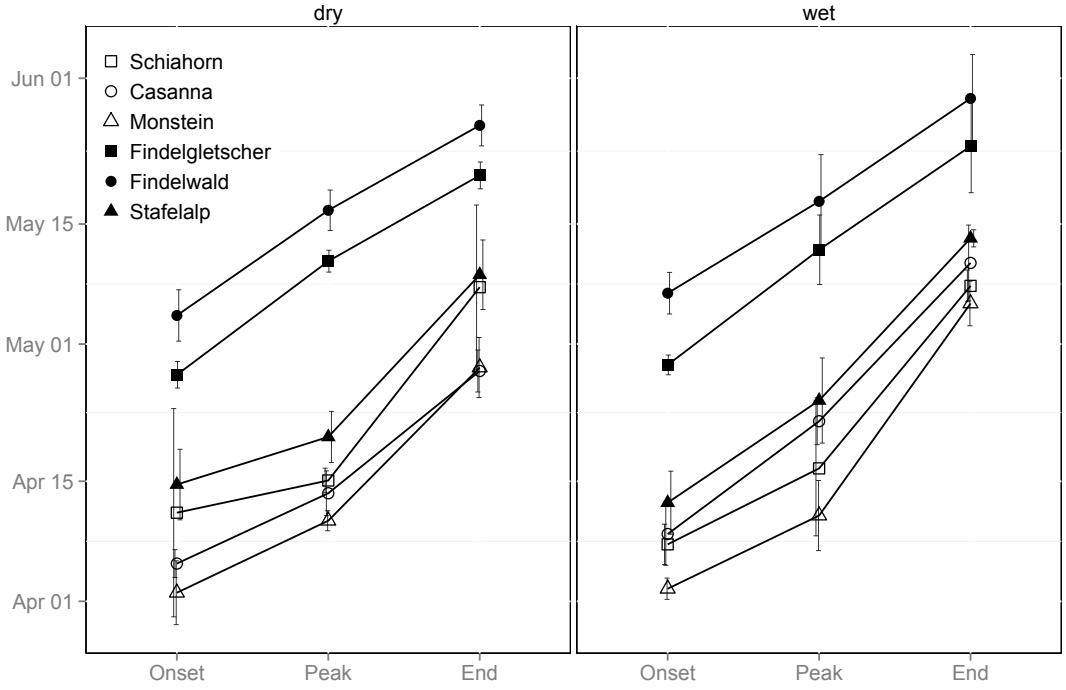

Fig. 1: Flowering phenology of six alpine populations of *Anthyllis vulneraria* in the common garden of the Botanical Institute in Basel in the two soil moisture treatments. Open symbols represent populations from Davos (Schiahorn, Casanna, Monstein), and closed symbols represent populations from Zermatt (Findelgletscher, Findelwald, Stafelalp). Error bars denote one standard error of the mean based on individual variation within population.

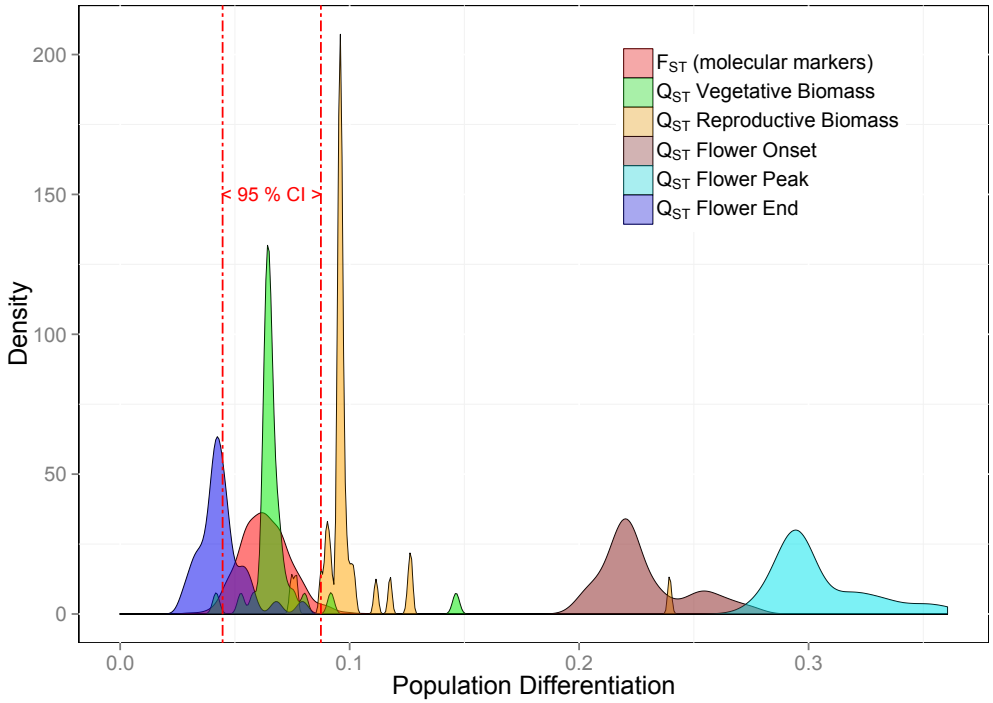

Fig. 2: Probability density distributions of F_{ST} and Q_{ST}'s for biomass and phenological traits. 10^3 replicate values of F_{ST} were generated by a non-parametric bootstrap. F_{ST} is based on microsatellites. Distributions of Q_{ST}'s for this plot consist of only 36 replicates per trait, which were generated by jackknifing over seed families. Vertical lines indicate the 95 % confidence interval of the estimate of F_{ST}. 95% confidence intervals of Q_{ST}'s of onset and peak of flowering do not overlap with the 95 % confidence interval of the estimate of F_{ST} and therefore reveal a signature of past selection.

Effects of treatment on biomass allocation and reproductive phenology

Regions responded differently in their aboveground biomass to the soil moisture treatment (significant *region x treatment* for vegetative and reproductive biomass, and reproductive allocation; Table 3). Contrasts of the treatments in each region revealed that vegetative biomass decreased in Zermatt under lowered soil moisture (p=0.003), while it was not significantly affected in Davos (p=0.93; Fig. 3). Concerning reproductive biomass, contrasts of the treatments in each region revealed that both the Davos and Zermatt regions were significantly affected by lowered soil moisture (p=0.04; resp. p<0.01), but the Zermatt region more strongly so. However, Figure 3 suggests the *region x treatment* for reproductive biomass was largely driven by the two geographically adjacent populations of Findelgletscher and Findelwald, which responded with a strong decrease in reproductive biomass to the drought treatment. The third population from Zermatt (Stafelalp) reacted very similarly to the Davos populations with a small decrease in reproductive biomass (Fig. 3). Overall, the population-level decrease in reproductive biomass in response to drought was proportional to the mean reproductive biomass across both treatments (Pearson's product moment, t=4.81, df=4, P<0.01). There was no correlation of reproductive allocation with vegetative biomass across all populations at the level of seed family (Pearson's product moment, t= 1.75, df=34, P>0.09), and therefore no indication of a trade-off between reproductive and vegetative biomass in the studied populations. No significant interactions of treatment with population or seed family were found.

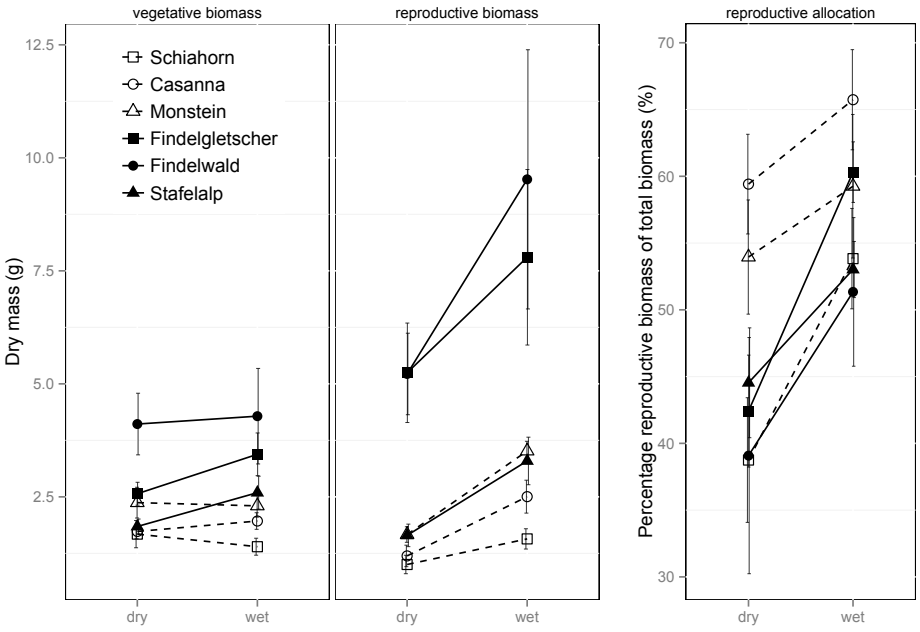

Fig. 3 Reaction norms of vegetative and reproductive aboveground biomass in response to two soil moisture treatments of six alpine populations of *Anthyllis vulneraria* in the common garden in Basel. Open symbols and dashed lines represent populations from Davos (Schiahorn, Casanna, Monstein), and closed symbols and solid lines represent populations from Zermatt (Findelgletscher, Findelwald, Stafelalp). Error bars denote one standard error of the mean based on individual variation within population.

Reduced soil moisture treatment had no consistent effect on the onset of flowering, but significantly advanced peak flowering and end of flowering of all populations by an average of 3 and 6 days, respectively. No significant interactions of treatment with population or seed family were found.

Discussion

The current study demonstrates considerable variation in reproductive phenology and aboveground biomass among six alpine populations of *Anthyllis vulneraria* sampled in two contrasting regions. Q_{ST}-F_{ST} comparisons suggest that divergent selection likely caused population genetic differentiation in onset and peak of flowering but not in biomass. Substantial amounts of variation in all measured traits are explained by family membership, indicating within-population genetic variation and sustained potential for future evolution. Soil moisture treatment had a significant effect on biomass traits, and resulted in a small but statistically significant forward shift of peak and end of flowering. No genetic variation in plastic responses to soil moisture was found within populations, neither for flowering phenology nor for biomass traits. Moreover, populations within each region did also not differ in their plastic response to drought, but across regions populations responded differently in vegetative biomass.

Variation in biomass allocation

There is good evidence that reproductive allocation increases along elevation (Fabbro and Körner 2004; Zhu et al. 2010). Since our populations are not spread along elevation very far and since we study only three populations per region, it is not surprising that we do not find differentiation beyond the neutral expectation in reproductive allocation across such short geographic distances as indicated by Q_{ST}-F_{ST} comparison.

Both vegetative and reproductive biomass were plastic in response to soil moisture availability suggesting that summer precipitation plays a role for growth and reproduction of alpine *Anthyllis vulneraria*. Drought stress in the alpine life zone is a phenomenon mostly reserved to special microhabitats such as extremely shallow or exposed substrates (Neuner et al. 1999), and tolerance to desiccation is often high (Körner 2003). However, reduced soil moisture - even if it does not cause problems with maintaining turgor - frequently leads to nutrient limitation and therefore reduced growth (Körner 2003). Interestingly, reduced soil moisture did not have a negative effect on vegetative biomass for populations from Davos. Previous results also show no effect or a slightly positive effect of drought for alpine grassland sites receiving high annual precipitation (Gilgen and Buchmann 2009). Drought stress therefore seems to be avoided in the Davos populations through slow growth resulting in lower total leaf surface area and consequently in lower transpiration. Whether this pattern is a genetic adaptation driven by regional differences in precipitation should be further investigated with a larger number of populations and measurements of in situ water availability. Statistical models and graphical inspection also showed that there is no significant within-population genetic variation in phenotypic plasticity in response to soil moisture, meaning that all seed families within a population responded similarly to soil moisture change. This is in line with the absence of divergent selection across populations as found in the Q_{ST}-F_{ST} comparison, because environmental heterogeneity in water limitation and

associated divergent selection are predicted to preserve genetic variation in plasticity (Via and Lande 1985). Although stabilizing selection across populations on trait means was not indicated by the Q_{ST}-F_{ST} comparison for biomass traits, stabilizing selection on reaction norms rather than on trait means might still be present and explain the absence of genetic variation in plasticity within populations.

Variation in reproductive phenology

Populations of *Anthyllis vulneraria* are differentiated in their reproductive phenology at all spatial scales from more than a hundred km to a few km. Snowmelt date was drastically advanced in our common garden compared to the natural sites because the garden is situated at much lower elevation. Genetic differences in photoperiodic sensitivity between populations, i.e. *G x E* in photoperiodic control, might therefore have become visible in our garden (Pigliucci 2003). Likewise *G x E* in vernalisation requirement might also contribute to the variation that was found in the common garden (Mendez-Vigo et al. 2013). Yet the strong forward shift in the phenology of all populations compared to the natural sites suggests strong insensitivity to photoperiod of all populations. Furthermore, we observed comparable differences in flowering time in an accompanying experiment with transplantations into the original field sites (Halil Kesselring, personal observation). Therefore it is more plausible, as the Q_{ST}-F_{ST} comparison suggests, that heterogeneous selection on onset and peak of flowering is the reason for the within-region population differentiation in these traits. Because a total of only 6 populations and a regional subdivision were used in our study, it is not feasible to correlate flowering dates with environmental variables at the sites of origin. Such correlations could strengthen the case for past and current adaptive evolution of flowering time and inform about selective agents. An emerging key environmental determinant of plant distributions in alpine habitats is spring frost (Bannister et al. 2005; Ladinig et al. 2013; Lenz et al. 2013; Briceño et al. 2014; Wheeler et al. 2014). The likelihood of spring frost at any elevation is largely determined by the date of snowmelt, which in turn is a function of winter precipitation, and topography. A thick snow cover in spring buffers temperature fluctuations and protects critical plant tissues from freezing damage due to very low temperatures. Reproductive structures of flowering plants are highly frost-susceptible and much less frost-tolerant than vegetative plant tissues (Neuner et al. 2013). Consequently, the timing of reproduction is expected to evolve so as to avoid periods with a high likelihood of frost. Sites with little snow accumulation during winter and relatively early snowmelt experience spring frost more commonly and should extend the pre-flowering duration. Similarly to spring frost, the emergence of pollinating insects of *Anthyllis vulneraria* can also potentially select for corresponding peak flowering times. As the activity of pollinating insects is strongly temperature-dependent, differences in elevation and exposition among populations could lead to divergent selection through pollinators (Kudo 1996). Flowering phenology is a trait particularly likely to be differentiated even over short geographical distances, because it is also a mechanism to reduce gene flow via pollen movement between individuals flowering at different times (Linhart and Grant 1996). There is a shortage of studies investigating whether populations of alpine plants at similar elevations experience divergent selection on flowering time by the local environments,

and future studies should test the link between snowmelt date, pollinator abundances, and flowering time.

The observed advances of peak and end of flowering in response to decreased soil moisture availability - although mild as they were - are in keeping with a strategy of quick reproduction under stressful conditions. This is a previously observed reaction of short-lived plants on short as well as evolutionary time-scales (Dunne et al. 2003; Franks 2011). Peak flowering date per seed family was not a function of the reproductive biomass (ANCOVA, F=1.25, p=0.27). Hence, it is unlikely that the advanced dates of peak and end of flowering are merely the result of decreased biomass. Likewise, the time between re-establishment of the treatment in the second growing season to onset of flowering was not correlated to plasticity in flowering onset either, meaning that later-flowering populations were not more plastic. Therefore, the absence of a plastic response of flowering onset to soil moisture is unlikely the result of the suspension of the treatment during winter. Advanced peak and end of flowering therefore potentially reflect an adaptive drought escape strategy in *Anthyllis vulneraria*. No variation in the response of the flowering phenology to soil moisture availability was indicated by statistical analyses, neither at the among-population nor at the within-population level (Tab. 3). As theory predicts that genetic variation in plasticity should be preserved under conditions of heterogeneous selection (Via and Lande 1985), one might conclude from these results that variability in soil moisture leading to heterogeneous selection on flowering phenology does not exist at the scale at which populations were sampled in this experiment (< 20 km within regions). Alternatively, evolutionary constraints or stabilizing selection on reaction norms may be present.

Accuracy of Q_{ST} and F_{ST} estimation

The method of comparing Q_{ST} to the neutral expectation using F_{ST} has been scrutinized, because both indices are not without problems (e.g. McKay and Latta 2002; O'Hara and Merila 2005). The accuracy of the estimation of Q_{ST} depends on how well we can separate additive genetic variance (V_A) within and between populations from environmental effects, maternal effects, and non-additive genetic effects such as dominance. Since we have largely reduced environmental variation by raising all plants in a common garden and controlling for soil moisture availability to our best ability, direct environmental effects should be minimal in our study (Leinonen et al. 2008). Indirect environmental variation can still occur through maternal effects in our design as we used maternal half-sibs to estimate V_A. However, maternal effects in plants have so far almost exclusively been found to affect only early life-history stages and to diminish over time (Bischoff and Müller-Schärer 2010 and references therein). As our plants were in the second growing season when traits were measured, maternal effects might not have had a considerable effect on the outcome. Furthermore, we have included early leaf size as co-variate in the analyses, a method commonly used to control for maternal effects (Scheepens and Stöcklin 2013). We used the phenotypic resemblance of open-pollinated half-sibs to assess V_A, a method that confounds additive with non-additive genetic effects such as dominance. However, non-additive effects always cause a downward bias in estimating Q_{ST} (Lynch and Walsh 1998), and therefore render tests of $Q_{ST}>F_{ST}$ conservative. As we found no Q_{ST} smaller than F_{ST}, this bias is unlikely to affect our conclusions. Finally, F_{ST} has been

hotly debated as an accurate measure of neutral population differentiation and the molecular markers used to assess it are criticised (e.g. Hedrick 2005; Jost 2008). In this study we used microsatellites, which are notorious for having a high mutation rate resulting in lower estimates of F_{ST}. Indeed, Jost's estimate of differentiation was more than twice as big as F_{ST}. However, since this difference is still mild compared to many previous microsatellite studies, and since far less than 1 private allele per locus and population was found (results not shown), we suspect that our microsatellites do not have an exceedingly high mutation rate and are therefore suitable for comparisons of Q_{ST} with F_{ST} (Edelaar and Björklund 2011). Furthermore, Jost's estimate of differentiation was still smaller than both Q_{ST}'s concluded to be significantly larger than F_{ST}. Consequently, if our F_{ST} falsely underestimates population differentiation at neutral marker sites, then this would mostly affect our conclusions that none of the traits is under stabilizing selection across populations. In summary, we are confident in the accuracy of the results with the exception of underestimating stabilizing selection across the Alps in plant size. Nonetheless, we caution the reader against taking Q_{ST}-F_{ST} comparisons as definitive proof for the presence or absence of selection.

Conclusion

Our results suggest that the timing of onset and peak of flowering has been under divergent selection among populations of alpine *Anthylllis vulneraria*. Populations were differentiated in onset and peak of flowering up to 5 weeks across regions and more than 2 weeks within regions when grown in the common garden. Analyses suggest that differentiation resulting from selection occurs even at spatial scales < 20 km and we hypothesize it is the result of temperature differences at the population sites resulting in divergent snowmelt and pollinator conditions. We found ample genetic variation within populations for all traits, supporting the idea that future adaptations in flowering phenology and reproductive allocation to novel conditions are possible. However, genetic variation in phenotypic plasticity in response to soil moisture availability was absent for all traits studied. This indicates either the absence of significant heterogeneity in soil moisture across populations, or stabilizing selection on reaction norms across populations, or in the case of flowering phenology might be due to the low overall plasticity in flowering phenology in response to soil moisture.

Supplementary Data

Additional supporting information is available in the online version of this article (see "supplementary material") and contains the following:
1_Map.pdf, 2_SoilMoisture.pdf,
3_Temperature. Pdf, RawData.xls.

Acknowledgements

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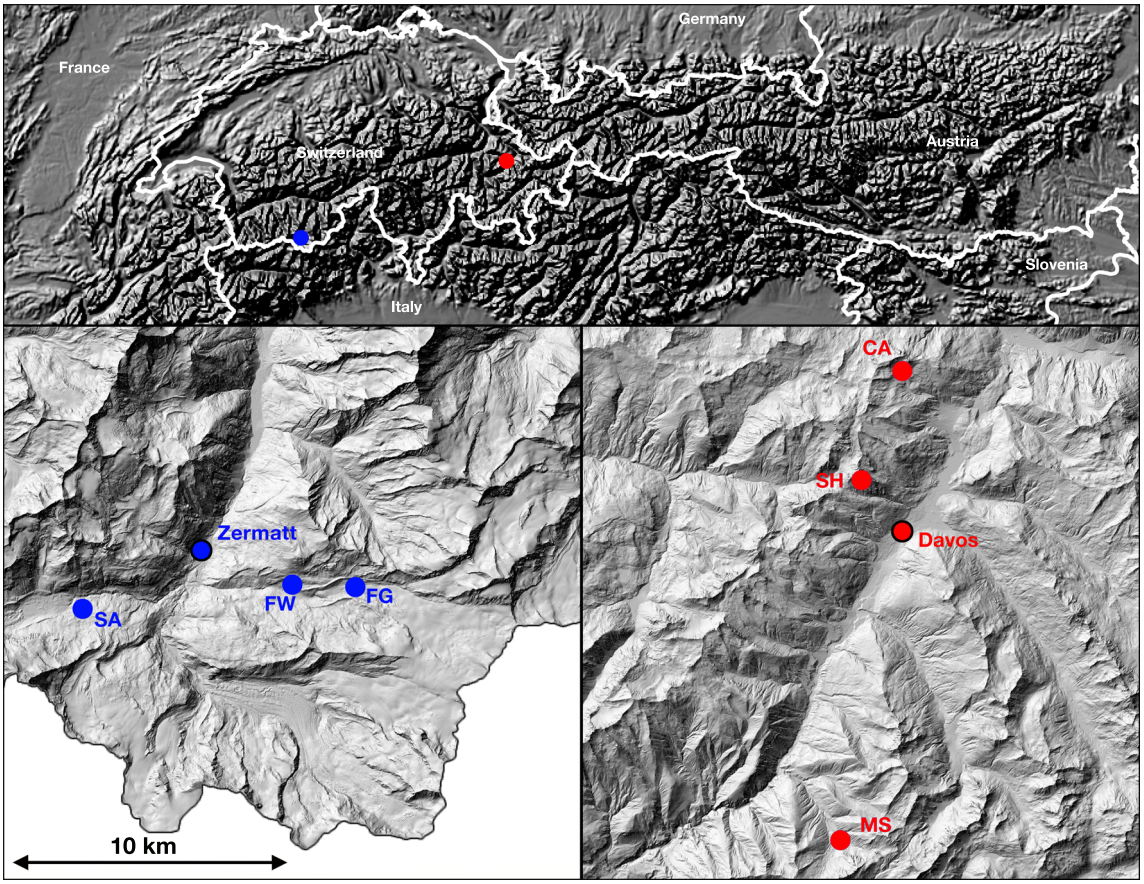
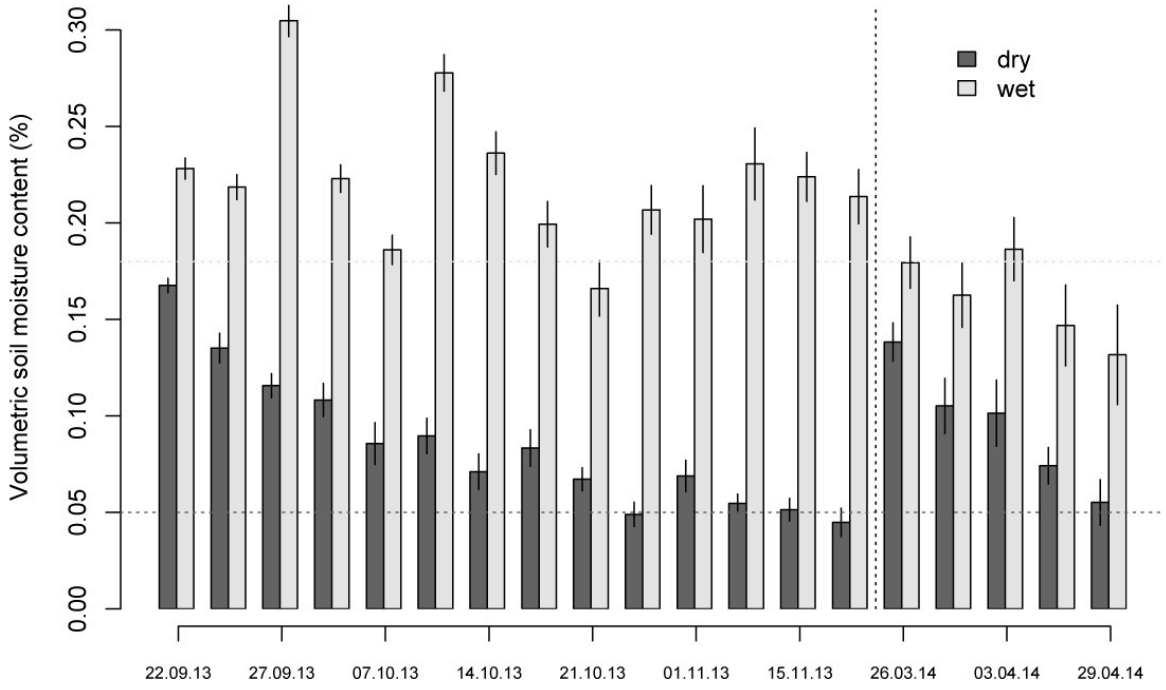
Supplementary Data

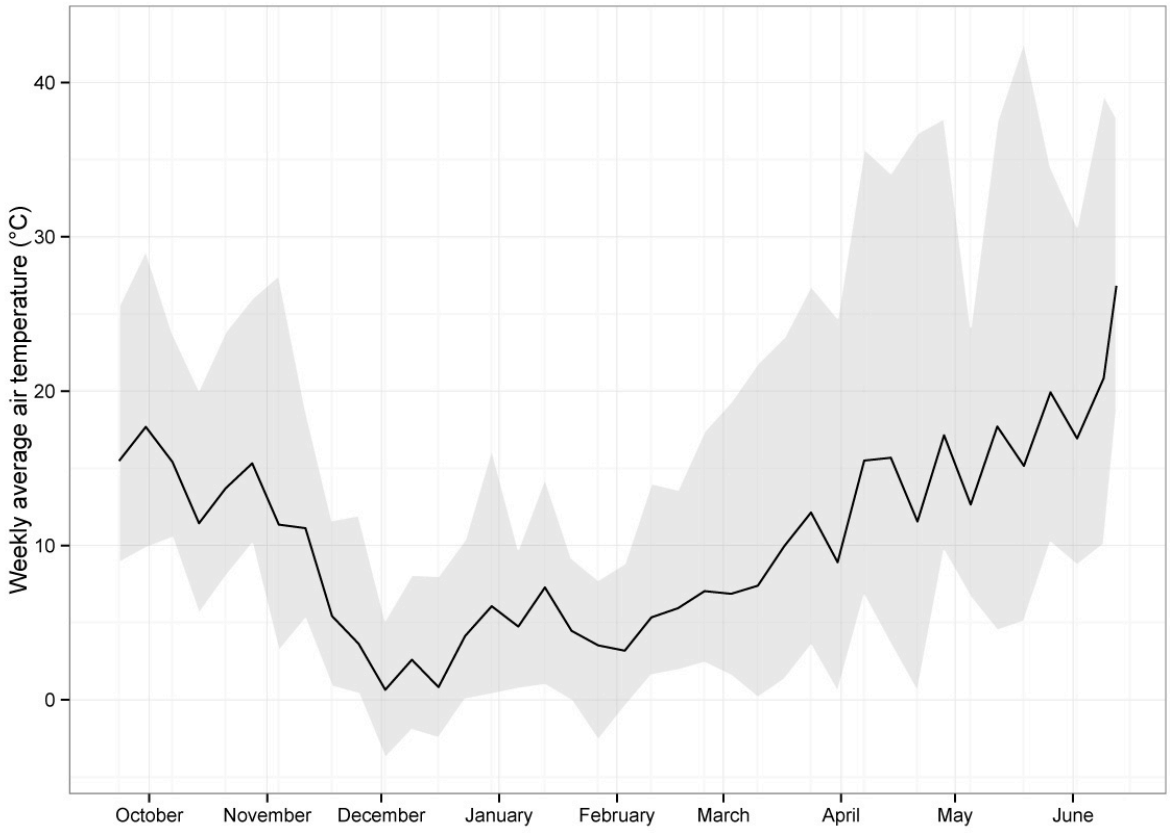
Online Resource 1 Hill shade maps of European Alps showing the 6 sampled populations. The top panel map is derived from the global digital elevation model of the European Environment Agency, and the two bottom panels are copyrighted by swisstopo, 3084 Wabern, Switzerland (Swiss ALTI^{3D}). The scale only applies to the bottom panels.



Online Resource 2 Volumetric soil moisture measurements were taken twice per week at random intervals and at different times after watering on 10 haphazardly selected individuals per treatment. Wet and dry plants were watered when the average moisture content undercut 18 % and 5 %, respectively as indicated by the horizontal lines. The vertical line indicates the suspension of the treatment during winter. Wet plants were given between 100 ml and 190 ml of water and on each watering, and dry plants between 60 ml and 100 ml, depending on water status.



Online Resource 3 Weekly average (black solid line) and weekly maximum and minimum (grey shade) air temperatures for the duration of the experiment.



Chapter 5

Evidence of local adaptation to fine- and coarse-grained environmental variability in *Poa alpina* in the Swiss Alps

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Evidence of local adaptation to fine- and coarse-grained environmental variability in *Poa alpina* in the Swiss Alps

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Abstract

• In the alpine landscape, characterized by high spatiotemporal heterogeneity and barriers, divergent selection is likely to lead to local adaptation of plant populations either through adaptive genetic differentiation or phenotypic plasticity. The relative importance of these processes has rarely been investigated in relation to the spatial scale of environmental heterogeneity. In this study we used reciprocal transplantation experiments of populations across nearby and distant field sites to shed light on these complementary processes.

• We reciprocally transplanted populations of the widespread alpine grass, *Poa alpina*, within and across regions in the Swiss Alps. We inferred local adaptation at the meta-population level by comparing fitness of plants transplanted to their site of origin, and to nearby or distant novel sites. Additionally, we measured specific leaf area (SLA) and performed selection analyses to investigate directional selection on mean trait values at the different field sites, and on the degree of plasticity of this trait to assess if plastic responses were adaptive. In parallel, all populations were genotyped with microsatellite markers to assess neutral molecular differentiation.

• Molecular differentiation was high among populations within and among regions, indicating restricted gene flow among *P. alpina* populations. Reproductive biomass was highest in individuals grown in their region of origin, revealing local adaptation to coarse-grained environmental variability. Similarly, inflorescence height, associated with reproductive biomass, reflected adaptation to fine- and coarse-grained environmental variability. Furthermore, we found evidence that plasticity in SLA across coarse-grained habitats was correlated with plant fitness, suggesting that plasticity in this trait is adaptive.

• Our results revealed adaptive genetic differentiation between *P. alpina* populations in the Swiss Alps reflecting local adaptation. Furthermore, high phenotypic plasticity in SLA contributed to the maintenance of fitness homeostasis across habitats. Hence, adaptive genetic differentiation and phenotypic plasticity play a complementary role for adaption of *P. alpina* to environmental heterogeneity in the Swiss Alps, and may both be critical to mitigate local extinction risk under rapid climate change.

Keywords: adaptive potential, genetic differentiation, phenotypic plasticity, plant-climate interactions, reciprocal transplantation experiment, spatial scale, sympatric vs. allopatric contrast.

Introduction

Intraspecific phenotypic variation among plant populations can arise from genetic drift, adaptive genetic differentiation, or passive and adaptive phenotypic plasticity (Van Tienderen, 1991, Van Tienderen, 1997). In widespread species, populations are often distributed across diverse habitats generating divergent selective pressures resulting in adaptive genetic variation among populations that maximizes fitness in local habitats (i.e. local adaptation; Lande and Arnold, 1983, Briggs and Walters, 1997, Kawecki and Ebert, 2004, Byars et al., 2007). In alpine environments, characterized by steep environmental gradients, strong spatio-temporal habitat heterogeneity, and high natural landscape fragmentation (Körner, 2003, Scherrer and Körner, 2010), local adaptation is likely to occur. Alternatively, heterogeneity across small spatial scales in these habitats could also favour adaptive phenotypic plasticity (Alpert and Simms, 2002). A highly plastic genotype producing a locally superior phenotype across habitats would rapidly become dominant, and would lead to phenotypic differentiation among habitats without underlying genetic differentiation (Kawecki and Ebert, 2004).

A central goal in ecological genetics has been to determine to what extent different phenotypes in contrasting environments result from genotypic differentiation, phenotypic plasticity or a combination of both (Van Tienderen, 1991, Van Tienderen, 1997, Conner and Hartl, 2004, Ghalambor et al., 2007, Gienapp et al., 2008). Theoretical conjectures relying on the scale of pollen and seed dispersal relative to the scale of environmental variability predict that local adaptation via genetic differentiation increases with geographic distance between populations, as a result of increasing environmental differences (i.e. coarse-grained environmental variation) and genetic isolation (Kawecki and Ebert, 2004, Baythavong, 2011, Volis et al., 2015). In contrast, genetic differentiation to environmental variability at a more local scale (i.e. fine-grained environmental variation) may be hindered by gene flow (Kawecki and Ebert, 2004), and phenotypic plasticity could instead be advantageous to accommodate environmental heterogeneity (Pigliucci, 2001, Sultan and Spencer, 2002, Baythavong, 2011). Accordingly, when attempting to understand how selection shapes plant responses to environmental heterogeneity, it is crucial to study patterns of local adaptation and phenotypic plasticity across different spatial scales characterized by fine- and coarse-grained environmental variation (Baythavong, 2011).

Furthermore, intraspecific genetic variation and phenotypic plasticity are two critical components of responses to changing environments (Byars et al., 2007, Nicotra et al., 2015), and can indicate the potential of plants to adapt to climate change (Kim and Donohue, 2013, Pluess et al., 2016). In the long term, intraspecific genetic differentiation can increase population persistence under climate change through dispersal of adaptive genes from other populations (Matter et al., 2014). This is particularly plausible in mountain systems where populations at lower elevations may provide adaptive genes to populations at higher elevations as climate change advances (Gonzalo-Turpin and Hazard, 2009, Matter et al., 2014). Alternatively, phenotypic plasticity can play a central role in the short-term adaptive potential of species by allowing the rapid accommodation of changes in environmental conditions (Richter et al., 2012), and can promote long-term adaptive evolution by buffering against

climate change (Price et al., 2003, Pigliucci et al., 2006, Nicotra et al., 2010). As such, characterizing genetic differentiation and phenotypic plasticity in alpine plant populations will help assess their adaptive potential to ongoing climate change (Till-Bottraud and Gaudeul, 2002, Byars et al., 2007, Pluess et al., 2016).

Reciprocal transplantation studies have long been used in alpine systems to investigate patterns of local adaptation to elevational gradients (Byars et al., 2007, Byars et al., 2009, Gonzalo-Turpin and Hazard, 2009, Hautier et al., 2009, Kim and Donohue, 2013, Scheepens and Stöcklin, 2013, Frei et al., 2014), snow cover (Stanton and Galen, 1997, Sedlacek et al., 2015) or land use type (Fischer et al., 2008). In most studies, local adaptation is inferred by comparing the fitness of "home vs. away" and/or "local vs. foreign" populations (Kawecki and Ebert, 2004, Bennington et al., 2012). While both these criteria are powerful tools to assess a "home-site advantage" of populations (Bennington et al., 2012), they can be confounded by intrinsic habitat or deme characteristics (Blanquart et al., 2013). This can be avoided by using a meta-population approach, which allows the removal of population and habitat effects before the assessment of local adaptation, by comparing the average fitness of a set of populations grown at their sites of origin (in sympatry, sensu Blanquart et al., 2013) and the average fitness of the same set of populations grown in novel sites (in allopatry, *sensu* Blanquart *et al.* 2013).

Along with increasing the statistical power to test for local adaptation through the meta-population approach (Blanquart et al., 2013), the "sympatric vs. allopatric" contrast can be enriched by partitioning the criterion according to the distance between transplantation sites (i.e. "sympatric vs. near-allopatric" and "sympatric vs. far-allopatric"). Thus this criterion is ideal when trying to investigate the spatial scale at which local adaptation operates (Banta et al., 2007, Richardson et al., 2014, Volis et al., 2015), and when attempting to understand the mutual role of adaptive genetic differentiation and phenotypic plasticity.

To do so, it is important to not only assess traits indicative of plant fitness but also key functional traits known to vary along environmental gradients (Liancourt et al., 2015). Leaf traits, in particular specific leaf area (SLA), are considered most indicative for plant resource management and stress tolerance (Lavorel and Garnier, 2002, Garnier et al., 2015), and strongly correlate with temperature, irradiance and soil water availability (Poorter et al., 2009, Scheepens et al., 2010). Phenotypic selection analyses, in which fitness is regressed against trait plasticity, can evaluate if trait plasticity is adaptive (Lande and Arnold, 1983, van Kleunen et al., 2000, Nicotra et al., 2015).

Here, we reciprocally transplanted populations of the alpine bunchgrass, *Poa alpina*, across original field sites in the Swiss Alps. This species was chosen for its wide distribution, its growth form ideal for transplantation of clonal genets, and because of its dual reproductive mode, relevant for gene dispersal distances across habitats. This study is among the first to use a meta-population approach and the "sympatric vs. near- and far-allopatric" criterion to investigate patterns of local adaptation across two spatial scales, between and within regions (i.e. fine- and coarse-grained environmental variation) on traits related to fitness (i.e. growth and reproduction). Additionally, we measured SLA at each field site and investigated potential selection on trait plasticity to infer on the adaptive value of phenotypic plasticity. In parallel, to assess

the genetic relatedness among the study populations, within and among region, genetic differentiation was analyzed using microsatellite markers. We specifically predict: (1) a fitness advantage in sympatric vs. allopatric (near and/or far) transplant combinations indicative of local adaptation; (2) that spatial scale determines the mechanism underlying local adaptation (i.e. natural selection favors phenotypic plasticity in fine-grained environmental variability, while coarse-grained environmental variability selects for genotypic differentiation).

Material and methods

Study species

The alpine meadow-grass, *Poa alpina* L. (Poaceae), is a perennial bunchgrass commonly distributed in arctic and alpine regions of the Northern hemisphere (Lauber and Wagner, 2001). In the European Alps, it occurs in natural sites up to 4200 m above sea level (a.s.l.) and in agricultural grasslands between 1400 and 2500 m a.s.l. (Conert, 1998, Aeschimann et al., 2004). This species has a broad ecological niche and grows in nutrient-rich meadows and pastures, but also in natural alpine grassland and on pioneer sites like scree fields and snow beds (Schröter, 1926, Conert, 1998). *Poa alpina* exhibits two reproductive modes: plants are either seminiferous seed producers (the majority are produced apomictically i.e. Steiner et al., 2012) or pseudoviviparous bulbil producers (Muntzing, 1940). The occurrence of seed-producing plants generally decreases with increasing elevation relative to bulbil-producing plants (Maurer, 2005, Fischer et al., 2011, Steiner et al., 2012). In alpine conditions, simulations found that seed dispersal via wind may exceed 1000 m (Tackenberg and Stöcklin, 2008). Moreover, this species is a polyploid complex, with highly variable chromosome numbers and common aneuploidy (Muntzing, 1980, Pierce et al., 2003).

Reciprocal transplantations

Location of transplantation sites

Population sites were chosen at two spatial scales: across a regional scale and across a local scale within regions. The two chosen regions in the Swiss Alps namely Davos and Zermatt differ in coarse-grained environmental conditions (notably climate). The distance between these two regions approximates 180 km. Davos is part of the Eastern Swiss Alps and situated in the Canton of Graubünden, while Zermatt is part of the Western Swiss Alps and located in the Canton of Valais. In Zermatt the annual average maximum, daily mean and minimum temperature is slightly higher than in Davos (Zermatt: 9.82, 4.23, -0.2, respectively and Davos: 8.7 °C, 3.5 °C, -1°C, respectively; MeteoSwiss). More importantly, Zermatt is a much drier region than Davos (MeteoSwiss, 2015), and receives almost half of the annual precipitation in rainfall and depth of snowfall of Davos (Zermatt: 639 mm and 263 cm, respectively and Davos: 1022 mm, 468 cm, respectively). Within each region, three populations differing in fine-grained environmental conditions (i.e. elevation, exposition; Table 1) were sampled at a maximal distance of 5 km of each other, implying that gene flow between these populations is restricted but not prohibited.

Experimental design

In September 2012 *P. alpina* plants were sampled from all 6 populations (Table 1). From each population, 10 healthy mother plants with at least 4 tillers were randomly sampled in the field at a minimum distance of 4 m from each other to avoid resampling

the same genotypes. In the greenhouse in Basel, Switzerland, tillers were individually potted in multitrays (54-pots of 4 cm Ø, 5 cm deep) filled with low nutrient soil (Anzuchterde, Ökohum GmbH, Herrenhof, Switzerland), watered regularly to water-holding capacity and fertilized once a month (Wuxal, Syngenta Agro, Dielsdorf, Switzerland). As plants grew and expanded, clonal tillers were regularly divided to obtain a total of 12 clonal offspring from each genotype and population (12 clones × 10 genotypes × 6 populations = 720 plants). In Spring 2013, plants were brought outside to acclimate to outdoor conditions before transplantations.

In July 2013, plants were transplanted into the field as soon as the snow had melted and the growing season had started. The reciprocal transplantations consisted of planting each population to its site of origin (sympatric), to one novel site within the same region (near-allopatric), and to one novel site in the foreign region (far-allopatric). Accordingly, each site received its local population, a foreign population from the same region and a foreign population from the other region. Combinations of foreign populations and sites were randomly chosen, but with the restriction that each foreign population was only used in one site within a region (Table 1). Each site received a total of 120 individuals, represented by 40 individuals per population (10 genotypes × 4 clonal replicates × 3 populations), which were planted directly into the local soil and watered once after planting to facilitate establishment. Individuals were planted following a stratified random pattern (i.e. alternating between individuals from each transplant combination) in rows of 10, with a minimal spacing of 20 cm between each other. Data loggers (Thermochrome iButton Device Model DS1921G, Maxim Intergrated Products, Inc., California, USA) were installed at each site (buried in the soil at a depth of 5 cm) to record hourly temperature at soil level.

Measurements

Initial number of tillers was recorded for each individual at time of transplantation. After two growing seasons, in October 2014, we assessed whether plants had survived and reproduced, counted the total number of tillers and the number of inflorescences, and measured the height of the tallest inflorescence. On the same date, aboveground biomass was harvested and separated into vegetative biomass and reproductive biomass. SLA was assessed at each transplant site (except at D_Schiahorn where stormy weather conditions made measurements logistically impracticable) and for each individual by taking three circular corings of 2.5 mm ∅ from different mature leaves, drying them at 60°C for 48h and weighing them together. SLA was then calculated as the fresh leaf area divided by the mean dry weight of the corings in $mm^2.mg^{-1}$ (Cornelissen et al., 2003). Vegetative and reproductive biomass were weighed separately after drying at 80°C for 72h.

Data analyses

All growth- and reproduction-related traits were analyzed with linear mixed-effect models (Crawley, 2007), using Type III sums of squares with the lmerTest package (Kuznetsova et al., 2013) for R (R Development Core Team, 2008). To test for local adaptation, we tested whether the means of the three different site x population combinations (i.e. sympatric, near-allopatric and far-allopatric) were significantly different from each other. To this end, we specified models including the fixed factors

site, population, the contrasts between sympatric, near- and far-allopatric transplant combinations, and the site × population interaction. Local adaptation was considered to be operating if (i) a sympatric vs. allopatric contrast was significant, and if (ii) sympatric transplant combinations outperformed allopatric ones (sensu Blanquart et al., 2013). To account for the replication of clonal tillers, the genotype of individuals (nested within populations) was included in all models as a random factor. The number of tillers at the time of transplantation was initially included in all models as a covariate to account for initial size, but was removed except in the models for the vegetative biomass and the total number of tillers where the covariate was significant. Vegetative biomass, reproductive biomass and the height of inflorescences were analyzed using a normal distribution with identity link function, while the total number of tillers and of inflorescences were analyzed using a Poisson distribution with log link function. An inspection of the distribution of residuals revealed no need for data transformation. Using lmerTest and its "rand" function, we report *F*-values and p-values for fixed effects and χ^2-values and p-values for random effects after Bonferroni correction for multiple comparisons ($p < 0.007$). Post hoc Tukey HSD tests were performed to detect significant differences among sympatric, near-allopatric and far-allopatric transplant combinations.

The proportion of surviving and reproductive individuals within each transplant combination (i.e. sympatric, near-allopatric, far-allopatric) was analyzed with generalized linear models using a binomial distribution with a logit link function.

Variation in SLA from populations transplanted within and across regions was used to assess the extent and adaptive value of plasticity in this trait. We used a linear mixed-effect model, including the fixed factors site, population and their interaction (site × population) to analyze SLA. Variance components were then calculated by fitting site, population and their interaction as random factors and extracting variances after Crawley (2007).

To assess potential phenotypic selection on SLA and on plasticity in SLA a phenotypic selection analysis would typically be performed for each site separately (Lande and Arnold, 1983, Conner and Hartl, 2004). However, our low sample size at each site prevents us to do so with sufficient statistical power. Hence, data from all sites was pooled, and a linear regression was performed to examine fitness as a function of SLA, site, and SLA by site interaction (SLA × site). The reproductive biomass of individuals was used as a fitness proxy. A significant SLA by site interaction would indicate that directional selection for specific SLA values varies in strength and/or direction among sites. Subsequently, we estimated the slopes of these correlations at each site as well as their significance to evaluate if SLA correlates positively or negatively with plant fitness at the different sites.

Finally, if directional selection on SLA values was found at certain sites, we proceeded to evaluate if phenotypic plasticity in SLA was adaptive (i.e. correlated with fitness), neutral or maladaptive. Standardized linear (i.e. directional) selection gradients were estimated as the partial regression coefficient of relative fitness on the standardized mean trait values of genotypes across transplant sites and on a standardized measure of plasticity across transplant sites (Relyea, 2002, Conner and Hartl, 2004). Relative fitness was calculated by dividing the reproductive biomass of genotypes by the mean across transplant combinations (i.e.

sympatric, near-allopatric, far-allopatric). Standardized mean SLA trait values were calculated across transplant combinations for each genotype. The degree of plasticity in SLA was calculated as the standardized phenotypic plasticity index (Pi_v = (max. mean – min. mean) / max. mean) across transplant combinations for each genotype following Valladares et al. (2006). This procedure was done for each of the two spatial scales studied here. Across the small spatial scales, we considered individuals transplanted within regions to nearby sites (i.e. in near-allopatry), and across the large spatial scale, we considered individuals transplanted across regions to the far-away site (i.e. in far-allopatry).

All analyses were performed on R version 3.0.2 software (R Development Core Team, 2013).

Molecular analyses

Sample collection

For the molecular genetic diversity analysis, leaf samples were taken from 60 plants in total, corresponding to 10 genotypes from the same 6 populations as used for the reciprocal transplantation study (Table 4). The samples, at least 2 cm long leaf parts, were dried with silica gel immediately after collection.

Genetic analyses

After milling the leaves in a Retsch MM400 mill (Retsch, Haan, Germany), DNA was extracted with the DNeasy plant mini kit (Qiagen GmbH, Hilden, Germany). We used illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) for microsatellite fingerprinting. Two of our previous *Poa alpina* studies (Maurer et al., 2005, Steiner et al., 2012) show details on PCR conditions of five microsatellite loci (CA1D4, GAC1, GA1C3, CA1F4, CAB12), horizontal gel electrophoresis of PCR amplicons, and ethidium bromide staining of Spreadex® gels as applied in the current study. Since *Poa alpina* is polyploid, we used a presence/absence (1/0) coding of the microsatellite banding patterns (see Rudmann-Maurer et al., 2007, Steiner et al., 2012). In a pre-analysis, we checked the repeatability of the PCR banding patterns in 4 individuals with replicates (i.e., two additional DNA extractions for each of the four individuals). For the five loci, we scored a total of 154 bands in the four individuals with 5 mismatching signals appearing in the repetition analysis, i.e. 5/154 = 0.03 = 3%. Accordingly, an acceptable replication rate of 97% was found.

Data analyses

We implemented the 1/0 data matrix into GenAlEx 6.2 (Peakall and Smouse, 2006) to identify multilocus genotypes (i.e. clones) and to perform an analysis of molecular variance (AMOVA) to partition genetic variability among regions, among populations within regions, and within populations. GenAlEx was also used to calculate the genetic differentiation indices, Φ, among regions and among populations within regions and to estimate their significance based on 999 permutations across the full data set.

Results

Reciprocal transplantations

As population PaD3 did not amplify to microsatellite PCR (see molecular variance below), we cannot exclude that another close *Poa* species might have been sampled by mistake at this site. Thus, to avoid any bias, we removed this population from our statistical analyses. Analyses were

consequently performed on 5 populations transplanted between 6 sites. Accordingly a total of 15 transplantations were analyzed, with 5 sympatric, 5 near-allopatric and 5 far-allopatric transplant combinations (600 individuals in total).

Site and population effects: All measured traits differed significantly among the six transplant sites indicated by significant site effects (Table 2, $p < 10^{-4}$ for all traits). Similarly, population effects for all measured traits were significant, or marginally so ($p < 0.10$), indicating genetic differences between populations (Table 2).

Local adaptation: The reproductive biomass differed significantly between sympatric and allopatric transplant combinations (Table 2; $F = 5.83$, $p = 0.003$). The reproductive biomass of far-allopatric transplant combinations was significantly lower relative to the reproductive biomass measured in sympatric and near-allopatric transplant combinations (Fig. 1c). Similarly, individuals transplanted back to their home site (sympatric transplants combinations) produced taller inflorescences than individuals from near- and far-allopatric transplant combinations (Table 2; $F = 5.35$ $p = 0.005$; Fig. 1a). Furthermore, the site × population interaction was significant indicating population differences among sites not related to local adaptation. For the number of inflorescences, a significant site × population interaction was found (Table 2; $F = 5.48$, $p = 0.001$). This result indicates that populations responded differently to environmental site conditions for this trait. Indeed, the number of inflorescences produced by individuals differed significantly between sites for population PAD1, PAZ2 and PAZ3. These populations either had a higher number of inflorescences when grown at their sites of origin (PADZ2), or the number of inflorescences decreased with increasing distance from the site of origin (Fig. 2; PAD1 and PAZ3). However, this pattern did not hold true on average for all population by site interactions, resulting in non-significant differences between sympatric vs. allopatric transplant combinations (Table 2; $F = 0.12$, $p = 0.8$; Fig 1b).

For the total number of tillers and the vegetative biomass the sympatric vs. allopatric contrast was non-significant for both of these growth-related traits (Table 2; $F = 2.63$, $p = 0.07$; $F = 2.79$, $p = 0.07$, respectively). Similarly, no significant site × population interaction was detected (Table 2) for these traits.

Phenotypic selection analysis

A significant site × population interaction was found for SLA ($F = 4.31$, $p = 0.01$) suggesting genetic variation in plasticity among populations, which is a condition *sine qua non* for an evolutionary response to selection on plasticity. The variance components analysis revealed that site effects (plastic changes) explained 72.2% of the trait variation found in SLA and the site × population interaction explained 27.8%, while the main population factor did not explain any trait variation.

Furthermore, a significant SLA × site interaction was found for the reproductive biomass (i.e. used as a fitness proxy) suggesting that directional selection for specific SLA values varies in strength among sites ($F = 4.32$, $p = 0.002$). While the slope of correlations between fitness and SLA was non-significant at most sites, a significant positive correlation between fitness and SLA was found at the Z_Rothorn site (Table 3; $\beta = 0.031$, $p = 0.002$). At this site, genotypes from the population of origin PaZ2, used as reference point, had a mean SLA of 10.78 ± 2.57.

Table 1: Location (site name with regional prefix: D for Davos and Z for Zermatt), geographic coordinates (latitude, longitude), elevation (m a.s.l.) and site characteristics of 6 *Poa alpina* populations sampled across the Swiss Alps. Pop, population abbreviation; Region, geographic region; *n*, sample size of individuals used in the transplantations; *g*, number of genotypes sampled for the transplantations; Site × pop, indicates which population was transplanted to which sites; Temp., mean temperature (°C) during July-October measured with data loggers at each site, reflecting the length of the growing season; Prec., amount of precipitation (mm) during July-October, reported for regions from MeteoSwiss; Exp., exposition of the transplantation sites.

Location	Pop	Region	Northing	Easting	Elevation	*n*	*g*	Site x pop	Temp.	Prec.	Exp.
D_Flüelapass	PaD1	Davos	46°44'49''	9°56'54''	2400	40	10	PaD1, PaD3, PaZ2	7.6	c.143	NW
D_Schwarzhorn	PaD2	Davos	46°43'41''	9°57'20''	2670	40	10	PaD2, PaD1, PaZ1	6.5	"	NE
D_Schiahorn	PaD3	Davos	46°49'01''	9°48'15''	2660	40	10	PaD3, PaD2, PaZ3	6.25	"	SE
Z_Schwarzsee	PaZ1	Zermatt	45°59'16''	7°41'51''	2760	40	10	PaZ1, PaZ2, PaD1	9.3	c. 59	SE
Z_Rothorn	PaZ2	Zermatt	46°01'02''	7°48'15''	2800	40	10	PaZ2, PaZ3, PaD2	8.9	"	NW
Z_Furi	PaZ3	Zermatt	45°59'26''	7°43'26''	2200	40	10	PaZ3, PaZ1, PaD3	11.6	"	SW

Table 2: Linear mixed-effect models for the responses in vegetative biomass, reproductive biomass, number of tillers, number of inflorescences, inflorescence height and in *Poa alpina* populations transplanted within and among regions. *F*- and *p*-values report on the fixed effects of site, population, the sympatric vs. near- or far-allopatric transplant contrast, and the remaining part of the site × population interaction. *P*-values marked in bold are significant after Bonferroni correction ($p < 0.007$). To account for the replication of clonal tillers, genotype was included in the model as a random factor, for which we report χ^2- and *p*-values. The number of initial tillers at time of transplantation was used as a covariate for the analysis of the number of vegetative tillers and the vegetative biomass of plants.

	Vegetative biomass			Reproductive biomass			Number of tillers			Number of inflorescences			Inflorescence height		
	Df	F / χ^2	*p*	Df	F / χ^2	*p*	Df	F / χ^2	*p*	Df	F / χ^2	*p*	Df	F / χ^2	*p*
Covariate	1	34.87	**$<10^{-4}$**	-	-	-	1	50.65	**$<10^{-4}$**	-	-	-	-	-	-
Site	5	37.01	**$<10^{-4}$**	5	47.21	**$<10^{-4}$**	5	30.74	**$<10^{-4}$**	5	27.81	**$<10^{-4}$**	5	96.8	**$<10^{-4}$**
Population	4	4.11	**0.002**	4	3.08	0.016	4	1.96	0.09	4	2.28	0.06	4	14.58	**$<10^{-4}$**
Symp. vs. Allop.	2	2.79	0.07	2	5.83	**0.003**	2	2.63	0.07	2	0.18	0.83	2	5.35	**0.005**
Site x population	3	2.07	0.10	3	4.92	**0.002**	3	1.74	0.15	3	5.48	**0.001**	3	8.34	**$<10^{-4}$**
Genotype	1	8.41	**0.004**	1	0.697	0.4	1	2.98	0.08	1	8.16	**0.004**	1	10.8	**0.001**

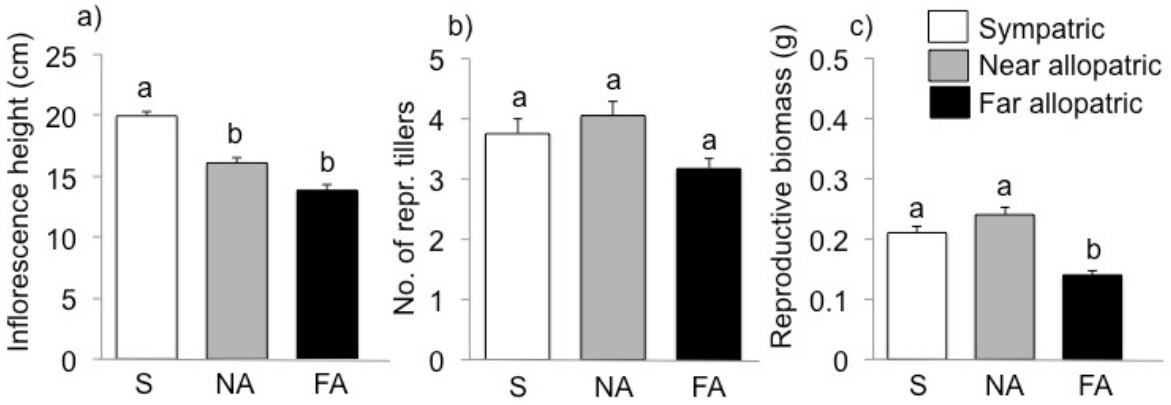

Fig. 1: Mean values ± SE for the inflorescence height (a), number of reproductive tillers (b) and reproductive biomass (c) for sympatric (S), near-allopatric (NA) and far-allopatric (FA) site × population transplant combinations. Results from the multiple comparisons between groups (post-hoc Tukey HSD test) are indicated as letters contrasts.

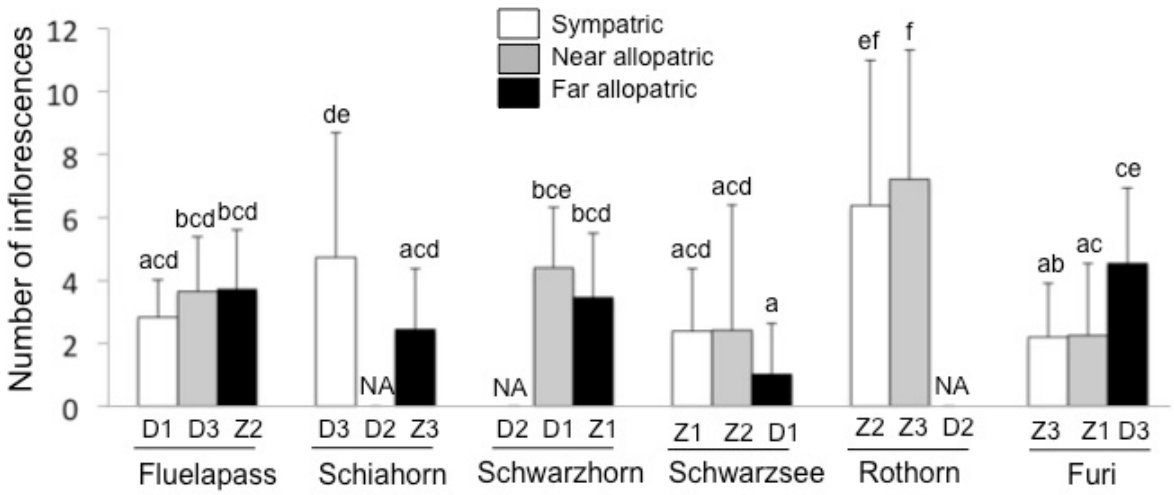

Fig. 2: Detailed mean ± SD of the number of inflorescences produced by each *Poa alpina* populations (PAD1, PAD2, PAD3, PAZ1, PAZ2, PAZ3) at each transplant site. Letters illustrate multiple contrasts (post-hoc Tukey HSD test) between site x population transplant combinations.

When genotypes from the near-by population PaZ3 were transplanted to this site, SLA increased to an average of 10.99 ± 1.84, in comparison to a mean of 8.85 ± 0.73 when these same genotypes were grown at their site of origin (Z_Furi). Plastic adjustments towards higher SLA values allowed foreign population to display a mean SLA close to the one of the home population, hereby increasing their fitness.

We further analyzed whether directional selection occurred on the degree of plasticity in SLA across transplant combinations (i.e. correlation between the phenotypic plasticity index and mean fitness of genotypes across sites). For comparisons across the small

spatial scale (i.e. across sympatric and near-allopatric transplant combinations) standardized selection gradients for the degree of plasticity were not significant (Pi_v: β = -0.10, p = 0.21). Hence, there was no evidence that selection favored phenotypic plasticity in SLA across the small spatial scale. However, at the larger spatial scale (i.e. across sympatric and far-allopatric transplant combinations), a significant and positive selection gradient was detected (Pi_v: β = 0.18, p = 0.006). Accordingly, genotypes showing a higher degree of phenotypic plasticity in SLA had an increased relative fitness across regions (Fig. 3).

Molecular variance

DNA extraction succeeded in five of the six populations, with following sample sizes: PAD1, N=10; PAD2, N=9; PAZ1, N = 10; PAZ2, N=10; and PAZ3, N=10 (see Table 4). In one population (PAD3), DNA of the leaf samples might have been degraded as microsatellite PCR did not work. Hence, the final 1/0 data matrix consisted of microsatellite signals of a total of 49 individuals of five populations. Among the 49 plants that were analyzed, we detected 62 banding positions among the five microsatellite loci, between 1 and 44 per locus. On average, individuals had 16.2 ± 2.17 allelic bands and their number did not significantly differ between populations. In total, we detected 31 multilocus microsatellite genotypes. No identical multilocus genotypes were found in population PaD2 and PaZ2, 8 unique genotypes out of 10 were found in PaZ3 and in PaD1, 5 out of 10 in PaZ1, and only 1 out of 10 in PaD3.

11% of molecular variance resided among regions, 25% among populations within regions, and 64% within populations (Table 4). The genetic differentiation indices, Φ, among regions and among populations within regions were significant. These results indicate high molecular regional and population differentiation, which is probably due to low gene flow among population.

Table 3: Mean ± SD SLA values of populations (code in parentheses; see Table 1) transplanted in sympatry, near- or far-allopatry to different sites (Flüelapass: D_Flüela.; Schwarzhorn; D_Schwh.; Schwarzsee; Z_Schws.; Rothorn: Z_Rotho.; and Furi: Z_Furi), and estimated slopes and *p*-values for the correlation between fitness (i.e. reproductive biomass) and SLA at each site. *P*-values are marked in bold when significant. Using the populations grown at their site of origin as reference points, we can assess if plastic adjustments in SLA of foreign populations (in allopatry) increased their fitness relative to when grown at their site of origin (in sympatry).

	SLA			Slope	
Site	Sympatric	Near-allopatric	Far-allopatric	b	*p*
D_Flüela.	11.46 ± 1.58 (PaD1)	12.45 ± 4.55 (PaD3)	10.96 ± 2.3 (PaZ3)	0.006	0.12
D_Schwh.	NA (PaD2)	15.10 ± 4.60 (PaD1)	12.35 ± 1.80 (PaZ1)	-0.007	0.18
Z_Schws.	15.76 ± 3.08 (PaZ1)	13.93 ± 4.92 (PaZ2)	11.16 ± 1.75 (PaD1)	0.006	0.38
Z_Rotho.	10.78 ± 2.57 (PaZ2)	10.99 ± 1.84 (PaZ3)	NA* (PaD2)	0.031	**0.002**
Z_Furi	8.85 ± 0.73 (PaZ3)	8.69 ± 1.04 (PaZ1)	9.61 ± 0.84 (PaD3)	0.007	0.30

*NA: missing values are explained by the removal of population PaD2 from analysis. Additionally, SLA could not be measured at the D_Schiahorn site because of harsh meteorological conditions.

Table 4: AMOVA results showing the molecular variance among regions, among populations within regions, and within populations (i.e. 2 regions, 5 populations) as well as genetic differentiation (Φ) among regions and among populations within regions and their significance based on permutation tests.

Source	df	SS	MS	Est. Var.	%	Φ	*p*
Among regions	1	62.72	62.72	1.25	11	0.11	0.005
Among pops within regions	3	101.37	33.79	2.73	25	0.28	0.001
Within pops	44	307.90	6.99	6.99	64	-	-

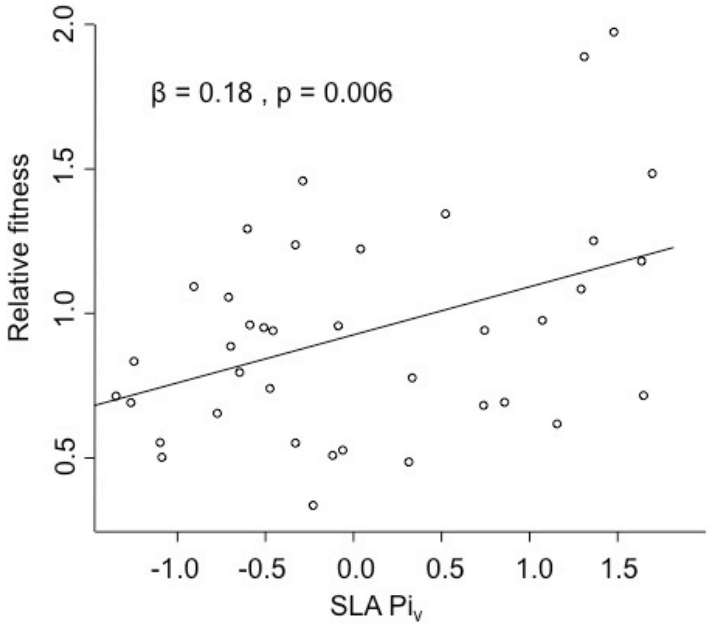

Fig. 3: Linear regression between relative fitness in terms of reproductive biomass and the SLA plasticity index (Pi_v) across sympatric and far-allopatric transplant combinations.

Discussion

The reciprocal transplantation of *P. alpina* populations across original field sites revealed pronounced effects of transplantation sites and populations of origin for all investigated traits indicating differences in site conditions and genetic differences amongst populations. For most traits, populations differed also in their responses to transplantation sites. These site × population interactions reflected a higher reproductive biomass and inflorescence height for individuals transplanted back into their site of origin (i.e. sympatric) relative to individuals transplanted to foreign sites (i.e. allopatric), indicating local adaptation in *P. alpina* populations in the Swiss Alps.

Local adaptation inferred from growth- and reproduction-related traits

Poa alpina populations showed a home-region advantage. Individuals transplanted back to their site of origin (in sympatry), or to a near-by site within the same region (in near-allopatry) had a higher reproductive

biomass. This signature for local adaptation in this fitness proxy is likely to be a result of the combination of increased inflorescence height in individuals from sympatric transplant combinations (Fig. 1a) and of decreased inflorescence number in individuals from half of the far-allopatric transplant combinations (Fig. 2). As both these traits directly contribute to the total reproductive biomass, we suggest that the similar patterns found in these traits reflect their association to reproductive biomass. Ultimately, these results indicate a higher reproductive output in individuals grown in their site and region of origin, and local adaptation of *P. alpina* populations distributed across the Swiss Alps.

The reproductive biomass significantly decreased in far-allopatric transplant combinations, indicating that local adaptation occurred in response to coarse-grained environmental variability and was probably related to regional differences in climatic conditions and length of the growing season. Indeed, Zermatt is a warmer and drier region than Davos (Table 1) and also receives less snow during winter, leading to earlier snowmelt and a longer growing season. As a result, the higher reproductive output displayed by individuals transplanted within their region of origin probably reflects genetic adaptation to the regional length of the growing season (Gugger et al., 2015). Furthermore, this result is in line with a number of studies that have found local adaptation to coarse-grained environmental variation (Galloway and Fenster, 2000, Banta et al., 2007, Volis et al., 2015) and supports the general consensus that local adaptation is more likely to occur over large geographic distances as a result of increasing environmental divergences and genetic isolation (Kawecki and Ebert, 2004, Hereford and Winn, 2008, Volis et al., 2015). Inflorescence height decreased in both near- and far-allopatric transplant combinations relative to sympatric ones, suggesting that plants grew better not only in their region of origin, but also at their site of origin within the region (i.e. small spatial scale). This result highlights that divergent selection can also lead to adaptive differentiation across relatively short distances in the alpine landscape (Gonzalo-Turpin and Hazard, 2009). While micro-geographic adaptation is often hindered by gene flow among nearby populations (Kawecki and Ebert, 2004, Richardson et al., 2014), we found a high molecular differentiation among nearby populations within the same region (Table 4). While previous estimations of dispersal distance of *Poa* seeds showed that seeds could potentially disperse across large distances (Tackenberg and Stöcklin, 2008), *Poa alpina* mainly reproduces via pseudoviviparous bulbils at high elevation (Steiner et al., 2012). Hence, the restricted gene flow, maintained through few apomictic seed producers, is unlikely to counteract population differentiation and local adaptation even at the small spatial scale studied here. This however emphasizes the importance of considering appropriate spatial scales relative to the dispersal distance of genes when studying patterns of local adaptation in widespread species (Baythavong, 2011).

The total number of tillers and the vegetative biomass differed between transplant sites and/or population of origin. However, no interaction was found between these factors and sympatric transplant combinations did not differ from allopatric ones. While these traits are often used to measure plant performance (Kawecki and Ebert, 2004) we argue that, in the case of *P. alpina,* they might not be the most suitable traits for detecting patterns of local

adaptation. Vegetative growth in *P. alpina* is mainly self-sustainable through photosynthesis in leaves and stems, while the production of reproductive structures is more costly (Watson, 1984). When flowering, *P. alpina* stops investing resources in growth of leaves and invests instead in reproductive shoots (Jürg Stöcklin, personal observation). Since the majority of individuals in our study reproduced (89%), differences in performance are probably limited in vegetative structures and mainly visible in reproductive traits. Furthermore, instead of measuring plant reproductive output at a certain point in time, studies should preferentially focus on lifetime fitness (Shaw et al., 2008, Shaw and Shaw, 2014). Ideally, germination rates, and seed-to-seedling transitions, which are critical components of life histories, should be investigated and integrated into local adaptation studies (Geber and Eckhart, 2005, Shaw and Etterson, 2012, Kim and Donohue, 2013, Shaw and Shaw, 2014, Wilczek et al., 2014).

Phenotypic plasticity in SLA

The variance components analysis revealed that most of the variation in SLA was explained by site effects (plasticity) and the site × population interaction (differences in plasticity between populations). Furthermore, directional selection favored high SLA values at the Z_Rothorn site, where a significant positive relationship was found between SLA and the reproductive biomass (Table 3). When foreign individuals were transplanted to this site, plastic adjustments occurred towards higher SLA values, approaching the mean displayed by the population of origin. An increase in SLA at the Z_Rothorn site, which had a northwestern exposition with lower light availability, could allow enhanced light interception, gas exchange and photosynthesis (Poorter et al., 2009). At the other sites, where no correlation between fitness and SLA was found, the combination of abiotic factors might have had confounding effects or might not have been strong enough to impose selection on this trait. Furthermore, one should also consider that SLA is a highly integrated measure indicative for various ecophysiological characteristics (Poorter et al. 2009), and therefore selection might also have acted on correlated traits not studied here (Conner and Hartl, 2004).

Interestingly, the degree of plasticity in SLA (Pi_v) was positively correlated with mean relative fitness of genotypes transplanted across regions (i.e. far-allopatric transplantations). Selection thus favored a high level of plasticity in SLA across coarse-grained habitats, a result that contrasts with our initial hypothesis. Indeed, phenotypic plasticity was expected to be favored across fine-grained habitats, as demonstrated empirically for the first time by Baythavong (2011). While our study may lack the statistical power to reveal selection for plasticity across fine-grained habitats, our results generally confirm that a high degree of phenotypic plasticity in this trait is adaptive (Nicotra et al., 2015). As such, adaptive phenotypic plasticity represents a means for *P. alpina* to maximize fitness in heterogeneous environments (Alpert and Simms, 2002, Baythavong, 2011).

Inferences about the adaptive potential of Poa alpina

Our AMOVA revealed high within-population molecular variance (Table 4), and in reciprocal transplantations we found highly significant genotype effects in most quantitative traits (Table 2), and high genetic differences among *P. alpina* populations (Table 2). These results highlight that

considerable genetic variation, which natural selection can act upon, is existent in this species widespread across the Swiss Alps, as previously shown by Rudmann-Maurer et al. (2007) and Fischer et al. (2008). Furthermore, the rigorous meta-population approach used here for analyzing reciprocal transplantations revealed a home-site advantage indicative of local adaptation. The evidence of adaptive variation among populations together with highly significant genotype effects suggests that there is a considerable adaptive potential to changing environmental conditions in *Poa alpina* (i.e. microevolutionary ability; Fischer et al., 2011). However, the question remains whether and to what extent adaptive genes of *P. alpina* populations can spread across the alpine landscape (Pluess et al., 2016). Considering the high genetic differentiation revealed among populations by neutral molecular markers gene flow seems limited even between nearby populations. Furthermore, whereas seeds from apomictic plants at lower elevation may possess adequate dispersal ability, bulbil-producing plants (i.e. clonal reproduction) at higher elevations may have much more restricted dispersal. Based on the previous elements, dispersal ability is probably low and gene flow restricted, suggesting that the effective adaptive potential of *Poa alpina* to rapid climate change may be compromised. In this context, adaptive phenotypic plasticity becomes even more crucial by allowing short-term responses to changes in environmental conditions, and buffering against climate change (Price et al., 2003, Nicotra et al., 2010). Moreover, when considering the high neutral molecular differentiation found among *P. alpina* populations and the polyploid complex of this species (Muntzing, 1980), we suggest that adaptive phenotypic plasticity might be critical for *P. alpina* to cope with novel environments or range boundaries (Alpert and Simms, 2002, Nicotra et al., 2010). Indeed, our study revealed that phenotypic plasticity in SLA was high across transplant sites and positively correlated with plant fitness when individuals were transplanted across regions. Therefore, we suggest that alongside with intraspecific genetic differentiation, phenotypic plasticity in this species' traits is critical to moderate the risk of local extinction when facing climate change.

Conclusion

Our study revealed adaptive genetic differentiation between *P. alpina* populations in the Swiss Alps reflecting local adaptation across fine- and coarse-grained habitats. While local adaptation of *P. alpina* populations across regions was expected due to increasing environmental variation and genetic isolation with increasing spatial scale, our results revealed that the pronounced fine-grained environmental variation in the alpine landscape, coupled with reproductive mode of this species, also lead to genetic differentiation across fine-grained habitats. Furthermore, our results confirm that phenotypic plasticity in SLA contributes to the maintenance of fitness homeostasis across heterogeneous environments. Hence, we conclude that both adaptive genetic differentiation and phenotypic plasticity act as complementary mechanisms allowing adaptation of widespread species to fine- and coarse-grained habitats, and may contribute to the short- and long-term adaptive potential of *P. alpina* to climate change.

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Chapter 6

High intraspecific phenotypic variation, but little evidence for local adaptation in *Geum reptans* populations in the Central Swiss Alps

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High intraspecific phenotypic variation, but little evidence for local adaptation in *Geum reptans* populations in the Central Swiss Alps

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Abstract

• The Alpine landscape is characterized by high spatiotemporal heterogeneity in environmental variables, such as climate and soil characteristics. This may lead to divergent selection pressures across plant populations and to local adaptation. *Geum reptans*, a widespread high-alpine clonal herb, has been the subject of several studies investigating phenotypic variation in populations across the Swiss Alps, yet so far, there is only little knowledge about local adaptation in this species from reciprocal transplantations across original field sites.

• Here, we reciprocally transplanted three populations of *Geum reptans* in the Central Swiss Alps, growing at close or far geographical distance from each other, and compared growth- and reproduction-related traits to investigate patterns of local adaptation. We further measured leaf morphological traits to assess potential selection at field sites, and quantified the relative importance of genetic vs. environmental variation (i.e. phenotypic plasticity) for all traits. Additionally, among and within population genetic differentiation was analyzed using microsatellite markers.

• Molecular diversity was high within populations, and molecular differentiation increased with geographic distance among populations, suggesting that gene flow is maintained at close range, but decreased with distance. Although extensive phenotypic variation was found across site × population transplant combinations, our study revealed little evidence for local adaptation in *G. reptans* populations. Plant traits also showed strong plasticity, as revealed by pronounced site effects, yet no direct linear selection was detected on leaf trait values within field sites.

• We suggest that the glacier forelands studied here, which are representative of the habitat of large *G. reptans* populations, are too similar in environmental conditions to lead to among population intraspecific differentiation in line with local adaptation. As *G. reptans* showed a great capacity to respond plastically to environmental conditions, we cautiously advocate that the evolution of phenotypic plasticity might have prevailed over genetic differentiation for the adaptation to the relatively narrow niche of this species.

Keywords: genetic differentiation, high-alpine clonal herb, molecular variation, phenotypic plasticity, reciprocal transplantation experiment.

Introduction

Alpine ecosystems are characterized by steep environmental gradients over short geographic distances (Körner, 2003) and a patchy microhabitat distribution (Scherrer and Körner, 2011), which offers numerous niches to alpine plant species (Aeschimann et al., 2004). These characteristics of the alpine landscape are often also associated with spatial isolation among populations and restricted gene flow (Stöcklin et al., 2009), which may allow for intraspecific population differentiation and local adaptation. Furthermore, at high elevation, plant life is challenged by low temperatures, late snow-melting, short vegetation periods and extreme weather events (Billings and Mooney, 1968, Körner, 2003). In this heterogeneous habitat, plants can adapt genetically to locally prevailing conditions (Byars et al., 2007, Gonzalo-Turpin and Hazard, 2009), and/or respond to spatiotemporal variability in environmental conditions via adaptive phenotypic plasticity (Sultan, 1995).

Intraspecific phenotypic variation resulting from genetic drift or natural selection (Volis et al., 2015) is common in widely distributed species (Bradshaw, 1984, Joshi et al., 2001, Banta et al., 2007) and has frequently been observed in alpine plant species (Pluess and Stöcklin, 2004, Giménez-Benavides et al., 2007, Byars et al., 2009, Gonzalo-Turpin and Hazard, 2009, Stöcklin et al., 2009, Frei et al., 2012). Moreover, strong phenotypic plasticity is likewise common in alpine species, and has been shown to provide a potential advantage for the persistence and survival of alpine species in a heterogeneous environment (Stöcklin et al., 2009, Frei et al., 2014). While the relative role of these two non-mutually exclusive strategies (i.e. adaptive genetic differentiation and plasticity), as well as the conditions for their evolution under divergent selection, are theoretically well-understood, empirical evidence is rather scarce (Baythavong, 2011, Hamann et al., 2016). The spatial grain size of environmental variation, defined by the degree of environmental variation as perceived by an individual plant across its dispersal distance, is likely to determine which of these mechanisms prevails (Alpert and Simms, 2002, Pigliucci et al., 2003, Kawecki and Ebert, 2004). Generally, adaptive genetic differentiation is expected when spatial grain size is coarse, whereas the evolution of phenotypic plasticity is expected when spatial grain size is fine (Baythavong, 2011, Richardson et al., 2014). As such, local adaptation via genetic differentiation is more likely to occur when gene flow is limited between populations, as is the case in naturally fragmented landscapes or in populations separated by great geographic distances (Kawecki and Ebert, 2004, Leimu and Fischer, 2008). Alternatively, adaptive genetic differentiation is unlikely to evolve when gene flow among populations is extensive, and depending on the spatial grain size, the evolution of phenotypic plasticity may prevail (Kawecki and Ebert, 2004). However, certain exceptions have been documented, such as adaptive genetic differentiation despite extensive gene flow, under strong micro-geographic divergent selection (Gonzalo-Turpin and Hazard, 2009, Richardson et al., 2014).

Local adaptation is also contingent on other factors such as plant mating system, longevity and clonality due to their effects on genetic diversity and the degree of genetic differentiation of populations (Galloway and Fenster, 2000, Kawecki and Ebert, 2004). Selfing, as opposed to outcrossing, reduces genetic diversity within populations, thereby

compromising future adaptive potential (Linhart and Grant, 1996). Similarly, clonality can limit the potential for local adaptation in case of limited sexual reproduction restricting genetic diversity within and among populations, but allows for plastic foraging among ramets (van Kleunen and Fischer, 2001). Additionally, clonal plants may be less locally adapted currently if long-lived genets reflect adaptation to past conditions (Leimu and Fischer, 2008, de Witte and Stöcklin, 2010). Nevertheless, at high elevation, clonal reproduction is common amongst alpine species, and is associated with benefits such as the ability to forage for resources, support the establishment of offspring, and buffer against environmental variation (Billings and Mooney, 1968).

Geum reptans L. (Rosaceae) is a long-lived clonal species occurring in high-alpine environments that reproduces sexually via strictly outcrossing flowers and vegetatively via clonal ramets on stolons. This species is an ideal system to study phenotypic variation and local adaptation, as it has been the subject of numerous prior investigations describing molecular and phenotypic variation, as well as gene flow among populations in the European Alps, and the relative importance of clonal vs. sexual reproduction (Pluess and Stöcklin, 2004, Pluess and Stöcklin, 2005, Weppler et al., 2006, Stöcklin et al., 2009, Frei et al., 2012). Findings from Weppler et al. (2006) suggested that the role of sexual reproduction was not restricted to the maintenance of genetic variation or long-distance dispersal, but played an equally important role for population growth as reproduction via clonal offspring (Weppler et al., 2006). Moreover, prior studies showed that genetic diversity within populations is high despite clonality (Ellstrand and Elam, 1993, Pluess and Stöcklin, 2004) and natural habitat isolation (Stöcklin et al., 2009). Direct measures of gene flow via seeds and pollen indicated the maintenance of considerable gene flow over short distances and low molecular differentiation among close populations (Pluess and Stöcklin, 2004). Furthermore, a glasshouse experiment revealed the great capacity of *G. reptans* to respond plastically to changes in environmental conditions, especially in its reproductive behavior (Pluess and Stöcklin, 2005). Finally, a common garden experiment with 20 *G. reptans* populations spanning all biogeographic regions of the European Alps revealed that phenotypic differentiation reflected the glacial history of this species shaped by founder effects and past selection, but also suggested adaptation to current climate conditions (Frei et al., 2012). However, to rigorously prove that adaptation to local conditions has occurred, reciprocal transplantation experiments across original field sites are necessary (Kawecki and Ebert, 2004), which have so far never been performed with this species.

Consequently, this study aimed at complementing previous ones, by investigating local adaptation via reciprocal transplantations of *G. reptans* populations growing at close or far geographical distances from each other in the Central Swiss Alps, and generally contributes to the body of empirical studies testing for local adaptation among alpine species. We investigated patterns of local adaptation in growth and reproductive traits by comparing the performance of sympatric and near- or far-allopatric site × population transplant combinations. We further measured leaf morphology traits known to be particularly plastic, yet not directly related to plant fitness (Frei et al., 2012), and investigated plastic responses to environmental site effects. For

all traits, we quantified the importance of genetic *vs.* environmental variation (i.e. phenotypic plasticity), and leaf traits were further used to investigate potential selection for mean trait values at each site. In addition, we analyzed among and within population genetic diversity and molecular differentiation using microsatellite markers.

We specifically investigate: (1) whether patterns of local adaptation are present amongst the studied populations of *G. reptans* (i.e. sympatric site × population transplant combinations outperform allopatric ones), (2) whether phenotypic plasticity is revealed in reproductive and leaf traits across sites, (3) if site-specific selection acts on leaf traits, and (4) whether genetic diversity within populations is maintained despite clonality and low molecular differentiation among close populations (i.e. because of gene flow over short distance).

Material and methods

Study species

Geum reptans L. is a long-lived high-alpine species belonging to the Rosaceae family. It is widespread in the European Alps and extends eastward to the Carpathian mountains (Conert et al., 1995). The species occurs above 2100 m above sea level (a.s.l.) up to 3800 m a.s.l., and grows typically on moraines in glacier forelands, and on moist scree fields and mountain ridges (Aeschimann et al., 2004). *Geum reptans* is an early-successional species colonizing virgin soils after glacier retreat and usually persists until interspecific competition becomes too strong (Weppler et al., 2006). The species grows in rosettes with dissected compound leaves. The number of leaflet pairs on a leaf usually ranges from c. 5 – 15. *Geum reptans* can reproduce vegetatively by forming new rosettes (ramets) at the end of long stolons, but can also reproduce sexually via seeds borne on a single-flowered stem. Both reproductive strategies are not mutually exclusive and seem to contribute equally to population growth (Weppler et al., 2006). The yellow flowers are proterogynous, pollinated by insects and c. 100 seeds are produced per flower (Pluess and Stöcklin, 2004). Seed dispersal spectra obtained from simulations showed that most seeds are dispersed across less than 10m, while long-distance seed dispersal over 100m and 1000m can occur for 0.015% and 0.005% of seeds, respectively (Pluess and Stöcklin, 2004, Tackenberg and Stöcklin, 2008).

Reciprocal transplantations

Three large *G. reptans* populations were chosen for this reciprocal transplantation experiment growing at close or far distance from each other in the Central Swiss Alps (Table 1). For clarity, we will refer to the populations using italic font and to the sites using capital letters. Two populations, abbreviated as *Flu* growing at Flüelapass (FLU) and *Dur* growing at Dürrboden (DUR), were located at relatively close proximity from each other (c. 5 km) near Davos (canton of Graubünden, Switzerland). A third population, abbreviated as *Mut* growing at Muttgletscher (MUT), was located at a larger geographic distance (c. 110 km from Davos) near the Furkapass (on the border between the canton of Uri and the canton of Valais, Switzerland). All three sites are glacier forelands but differed in elevation and exposition (Table 1). Soil temperature was recorded (as a proxy of smoothed air temperature; Körner and Paulsen, 2004) during the second growing season (July – October 2015) using one data logger buried in the soil at a depth of 5 cm at each site (Thermochrome iButton Device Model

DS1921G, Maxim Intergrated Products, Inc., California, USA). Mean temperature differed among sites when averaged over the time of measurement (Table 1). Precipitation records, obtained for each site from nearby meteorological stations (MeteoSwiss, 2015), also differed between sites when summed over the second growing season (July – October 2015; Table 1).

Table 1: Location, geographic coordinates (latitude, longitude), elevation (m a.s.l.) and site characteristics of 3 *Geum reptans* populations sampled in the Central Swiss Alps. Pop, population abbreviation (in italic font); *n*, sample size of individuals used in the transplantations; Temp., mean temperature (°C) averaged from July-October 2015, indicative of the length of the growing season, measured with data loggers at each site; Prec., summed amount of precipitation (mm) from July-October 2015, as obtained from the nearest weather stations to our sites (Weissfluhjoch for FLU, Davos for DUR, Gütsch ob Anderatt for MUT, respectively; MeteoSwiss 2015); Exp., exposition of the slopes of the transplantation sites.

Location	Pop	Latitude	Longitude	Elevation	*n*	Temp.	Prec.	Exp.
Flüelapass (FLU)	*Flu*	46°44'54''	9°56'54''	2400	40	8.85	570	NE
Dürrboden (DUR)	*Dur*	46°42'29''	9°56'12''	2290	40	10.22	525	NE
Muttgletscher (MUT)	*Mut*	46°33'27''	8°24'39''	2480	40	7.39	465	NW

In September 2013 *G. reptans* populations were sampled from all three sites. For each population, 40 healthy mother plants were randomly chosen and three viable stolons with rosettes (ramets) were collected from each of these individual. A minimum sampling distance of 5 m between mother plants was respected to minimize the risk of resampling genotypes (Pluess and Stöcklin, 2004). Rosettes were kept in plastic bags and stored at 4 °C in the dark for a maximum of two days until they were planted in the greenhouse (Botanical Institute, Basel, Switzerland) in separate pots 7 x 7 x 8 cm filled with potting soil (Containererde, Ökohum GmbH, Herrenhof, Switzerland). Rosettes were grown for nine months in the greenhouse, watered regularly to soil capacity, fertilized once a month (Wuxal, Syngenta Agro, Dielsdorf, Switzerland), and treated once with an insecticide (Spruzit®, Neudorff GmbH, Germany) to control infestations of Aphidoidae and Aleyrodidae. Four weeks before transplantation to field sites, plants were placed outdoors (Botanical Garden, Basel, Switzerland) for acclimation.

In July 2014, plants were reciprocally transplanted into field sites as soon as the snow had melted and the growing season had started. For each population, one ramet per genet was transplanted to each of the three sites. Each site thus received a total of 120 individuals, represented by 40 individuals per population (40 genets × 3 populations). Due to the MUT site being far away from the two relatively nearby sites near Davos, transplantation resulted in 3 sympatric (i.e. populations transplanted back to their site of origin), 2 near-allopatric (i.e. populations transplanted to a site at close proximity) and 4 far-allopatric (i.e. populations transplanted to a site at far distance) site × population transplant combinations. Individuals were transplanted into the local soil, in a patch within the natural populations, and experienced local intra- and inter-specific competition, reflecting natural conditions. Tagged individuals were planted in rows of 10, alternating between populations, with a

minimal spacing of 20 cm between each other, and were watered once after planting to facilitate establishment.

Initial number of leaves was counted immediately after transplantation. After two growing seasons, in October 2015 we assessed whether plants had survived and reproduced. Number of leaves was counted on surviving individuals, and the number of flowers and/or stolons was counted for reproductive individuals. The total number of reproductive meristems was calculated for each individual by adding individual number of flowers and stolons. To assess the relative importance of clonal vs. sexual reproduction, we calculated the clonality of each individual as the proportion of stolons on all reproductive meristems. For each individual, we identified the longest leaf, measured its length and width, and counted the number of leaflets. As an indicator of its leaf shape, (i.e. also called leaf aspect ratio) we calculated the ratio between leaf length and leaf width. Degree of leaf dissection was estimated by dividing the number of leaflets by the leaf length. SLA was assessed for each individual by taking four circular corings of 5 mm ∅ from different mature leaves (avoiding veins), drying them at 60°C for 48h and weighing them together. SLA was then calculated as the fresh leaf area divided by the mean dry weight of the corings (Cornelissen et al., 2003). Aboveground dry mass was harvested and dried at 80°C for 72h before weighing.

Data analyses

All traits were analyzed with generalized linear mixed-effect models (Crawley, 2007), using Type III sum of squares with the lme4 (Bates et al., 2015) and lmerTest packages (Kuznetsova et al., 2013) for R. To test for local adaptation in survival, growth- and reproduction-related traits using the sympatric vs. allopatric contrast, we tested whether the means of the three (sympatric, near-allopatric and far-allopatric) distributions significantly differed from each other. To this end, we specified models including the factors site, population, and the contrast between sympatric, near- and far-allopatric transplant combinations (Blanquart et al., 2013). Local adaptation was considered to be operating if (i) the sympatric vs. allopatric contrast was significant, and if (ii) sympatric transplant combinations outperformed allopatric ones (Blanquart et al., 2013). The replication of genets within populations was accounted for by including this factor in the models as a random factor. The initial number of leaves recorded at the time of transplantation was included in the model as a covariate to account for effects of initial plant size. This factor was, however, non-significant and therefore removed from the model for all traits except the final number of leaves.

For traits related to leaf morphology (i.e. leaf shape, leaf dissection and SLA), we analyzed if plastic responses were displayed in response to environmental conditions at field sites and if these responses differed between populations. To this end, we specified models testing for differences between sites and populations, the interaction between site and population, and included genets as a random factor in the model.

The proportion of surviving, reproductive (clonal and/or sexual), and flowering individuals within each transplant combination (i.e. sympatric, near-allopatric, far-allopatric) was analyzed using a binomial distribution with a logit link function. The number of leaves, flowers, stolons and total number of reproductive meristems were analyzed using a Poisson distribution with log link function (zero-inflated for the number of flowers, stolons and total

reproductive meristems). The remaining traits were assessed using a normal distribution with identity link function (Crawley, 2007). To normalize data and homogenize variance aboveground dry mass was log-transformed, count data (log+1)-transformed, and ratios (clonality, leaf shape, leaf dissection and SLA) arcsine-transformed (Crawley, 2007). We report *p*-values after Bonferroni correction (i.e. *p*-values multiplied for nine response variables) and *F*-values (for fixed effects) or χ^2-values (for random effects), the latter extracted with the "rand" function in lmerTest. Post-hoc Tukey HSD multiple comparison tests were applied in the multcomp package (Hothorn et al., 2014) to detect significant differences among site × populations transplant combinations.

Variance components were calculated for all traits by fitting site, population, their interaction and genets as random factors. We extracted variances using the "VarCorr" function from the lme4 package (Crawley, 2007).

Furthermore, as strong plastic effects were found in leaf traits, a follow-up selection analysis was performed to determine if environmental conditions at each field site selected for particular mean trait values. To do so, selection gradients were calculated by means of multiple linear regressions (Lande and Arnold, 1983). Leaf shape, leaf dissection and SLA site-specific trait values were standardized to a mean of zero and a variance of 1 prior to analysis. Relative fitness was calculated by dividing the number of reproductive meristems of each genet by the site-specific mean. Standardized linear (i.e. directional) selection gradients were estimated as the partial regression coefficient from the multiple regression of relative fitness on all standardized traits (Haggerty and Galloway, 2011). We report selection gradients β and *p*-values after Bonferroni correction (i.e. *p*-values multiplied for three response variables).

All the analyses were performed on R version 3.0.2 software (R Core Team, 2013).

Molecular analyses

Leaf samples were taken randomly from 20 out of the 40 sampled mother plants of each population *Flu*, *Dur* and *Mut* and immediately dried for DNA extraction using silica gel. Microsatellite marker development was performed by Ecogenics GmbH (Zurich-Schlieren, Switzerland), whose screening technique has previously been described in Kesselring et al. (2013). The 60 individuals were genotyped for nine microsatellite loci. A detailed description of microsatellite multiplex PCR in *G. reptans* can be found in Hamann et al. (2014). In brief, three multiplex PCRs were run. Multiplex I comprised primers for loci 015967, 011721, and 013998; multiplex II for loci 002235, 003651 and 011534, and multiplex III for loci 015615, 013198 and 007389. A fraction of the forward primers was fluorescent labeled with ATTO-dyes or FAM. Each multiplex PCR started with a denaturation step at 95°C for 15min, followed by 35 cycles of 94°C for 30sec, 56°C for 90sec, and 72°C for 60sec, with a final extension step at 72°C for 30min. Amplicons were loaded on an ABI3730 sequencer using an Eco500 size standard. Allele calling and crosschecking of genotypes was done with GeneMarker version 1.80 (SoftGenetics, State College, Pennsylvania, USA). Multiplex fingerprints in *G. reptans* have proven to be highly reproducible with an error rate of 1.4%. Nonetheless, binning of a few alleles was performed (see Table 1 in Hamann et al., 2014). The final table of genotypes was exported to GenAlEx 6.5 (Peakall and Smouse, 2006). GenAlex was used to check for identical multilocus genotypes among

sampled individuals, and to estimate the genetic diversity within populations, calculated as the unbiased expected heterozygosity (He; Nei, 1973). Additionally, the same software was used to perform an analysis of molecular variance (AMOVA) with 999 permutations to analyze partitioning of molecular variance among and within populations, and to calculate population pairwise F_{ST} values based on allele frequencies.

Results

Proportion of surviving and reproducing plants

On average, 85.8% of individuals survived from transplantation into field sites until harvest two growing seasons later. Survival was, however, independent of sympatric *vs.* near- and far-allopatric transplant combinations (F = 0.34, p = 0.92). Of the surviving individuals, on average 40.6% of individuals reproduced during the second growing season via sexual and/or vegetative meristems, yet this proportion was also independent of sympatric *vs.* near- and far-allopatric transplant combinations (F = 0.11, p = 0.69). The frequency of individuals producing flowers was low with an average of only 17.5%. Nevertheless, the proportion of individuals that flowered when transplanted to a distant site (i.e. far-allopatric) was lower compared to individuals transplanted back home or to a nearby site (F = 2.27, p = 0.03). Indeed, only 14% of individuals flowered when grown in far-allopatric transplant combinations against 20% and 29% in sympatric and near-allopatric ones, respectively.

Fitness-related growth and reproductive traits

No significant differences were detected across the sympatric vs. allopatric contrast for any of the studied growth and reproductive traits (Table 2), suggesting that these fitness-related traits did not differ between populations across transplant sites. However, highly significant site effects were detected for all fitness-related traits, except for the number of flowers and clonality (Table 2). Similarly, population effects were strong for aboveground dry mass and the number of stolons (F = 9.98, $p < 10^{-4}$, F = 8.64, p = 0.0018, respectively; Table 2). Site and population effects were pronounced for aboveground dry mass as population *Dur* grew best at the MUT site, even relative to the sympatric population *Mut* (Fig. 1a). Similarly, site effects were visible for the number of leaves, which was higher in population *Flu* and *Dur* when grown at the MUT site, relative to when grown at the FLU site (Fig. 1b). The number of flowers differed between genets (F = 7.67, p = 0.05; Table 2), and while the number of flowers produced by population *Flu* and *Dur* tended to be lower when grown at the far-away MUT site (Fig. 2a), this site effect was not significant after Bonferroni correction (Table 2). The number of stolons produced by individuals was particularly high in population *Flu* when grown at its site of origin (FLU), and relative to the population *Mut* when grown together at the FLU site (Fig. 2b). Similarly, site effects on the total number of reproductive meristems were pronounced for population *Flu* (Table 2), which produced a higher number of reproductive meristems when grown at its site of origin (FLU) relative to at the DUR site (Fig. 2c). Moreover, a genet effect was revealed for the total number of reproductive meristems (F = 8.11, p = 0.036; Table 2).

Table 2: Results of generalized linear mixed-effect models for the responses in growth- (aboveground dry mass, number of leaves) and fitness-related traits (number of flowers, number of stolons, total number of reproductive structures, and clonality) in *Geum reptans* populations transplanted across field sites. *F*- and *p*-values report the effects of site, population, the sympatric vs. allopatric contrast calculated as fixed factors. To account for the variation among genets within populations, this factor was included in the model as a random factor, for which χ^2- and *p*-values are reported. The covariate (i.e. number of initial leaves at time of transplantation) was significant only for number of leaves, and removed from models for the other traits. The *p*-values indicated in bold were significant after Bonferroni correction (at $\alpha = 0.05$; *p*-values multiplied by 9 for correction), *p*-values in italics were significant before Bonferroni correction, and non-significant *p*-values were truncated at 1 if >1 after correction.

	Aboveground dry mass (g)			Number of leaves			Number of flowers			Number of stolons			Total reproductive meristems			Clonality		
	Df	F/χ^2	*p*	Df	F/χ^2	*p*	Df	F/χ^2	*p*	Df	F/χ^2	*p*	Df	F/χ^2	*p*	Df	F/χ^2	*p*
Covariate	-	-	-	1	189.04	**$<10^{-4}$**	-	-	-	-	-	-	-	-	-	-	-	-
Site	2	16.95	**$<10^{-4}$**	2	39.80	**$<10^{-4}$**	2	3.99	*0.18*	2	10.32	**$<10^{-4}$**	2	12.15	**$<10^{-4}$**	2	0.58	1
Population	2	9.98	**$<10^{-4}$**	2	0.29	1	2	1.26	1	2	8.64	**0.0018**	2	4.24	*0.09*	2	3.21	*0.36*
Symp vs. Allop	2	0.93	1	1	0.90	1	1	1.78	1	1	0.46	1	1	0.05	1	1	0.92	1
Genets	1	1.56	1	1	4.22	*0.36*	1	7.67	**0.05**	1	0.82	1	1	8.11	**0.036**	119	0.62	1

Table 3: Results of generalized linear mixed-effect models for the responses in the leaf shape, leaf dissection and SLA in *Geum reptans* populations transplanted between field sites. *F*- and *p*-values report the effects of site, population, and the site × population interaction calculated as fixed factors. To account for the replication of genets within populations, this factor was included in the model as a random factor, for which χ^2- and *p*-values are reported. The *p*-values indicated in bold were significant after Bonferroni correction (at $\alpha = 0.05$; *p*-values multiplied by 9 for correction), *p*-values in italics were significant before Bonferroni correction, and non-significant *p*-values were truncated at 1 if >1 after correction.

	Leaf shape			Leaf dissection			SLA		
	Df	F/χ^2	*p*	Df	F/χ^2	*p*	Df	F/χ^2	*p*
Site	2	12.71	**$<10^{-4}$**	2	10.90	**$<10^{-4}$**	2	11.64	**$<10^{-4}$**
Population	2	8.91	**0.0009**	2	13.01	**$<10^{-4}$**	2	4.71	*0.081*
Site x population	4	1.49	1	4	0.49	1	4	1.86	1
Genets	1	0.97	1	1	14.4	**0.0018**	1	1.62	1

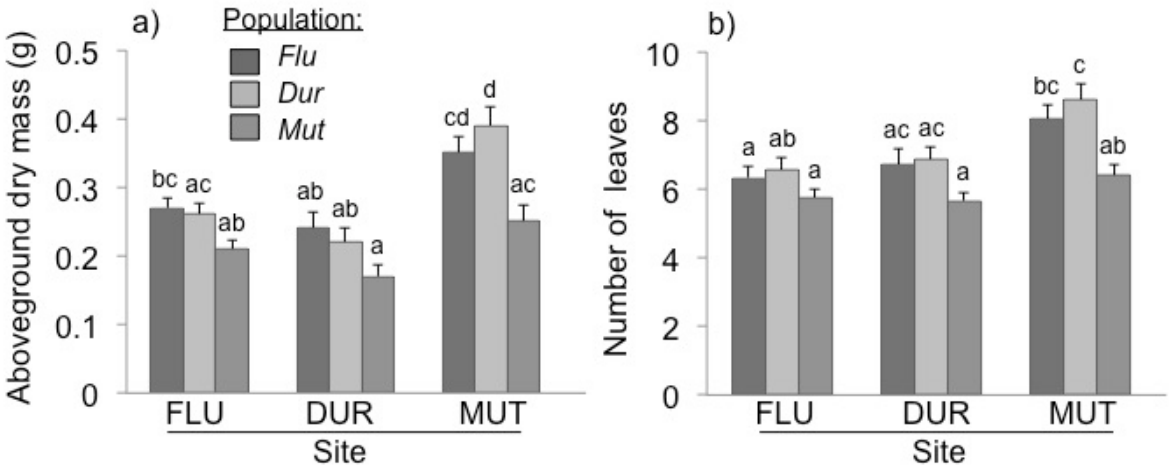

Fig. 1: Mean ± SE of growth-related traits: aboveground dry mass (a) and number of leaves (b) in *Geum reptans* populations (*Flu*, *Dur*, *Mut*) transplanted across three sites (Flüelapass: FLU, Dürrboden: DUR, Muttgletscher: MUT). Letters reflect multiple contrast results (post-hoc Tukey HSD test) between site × population transplant combinations.

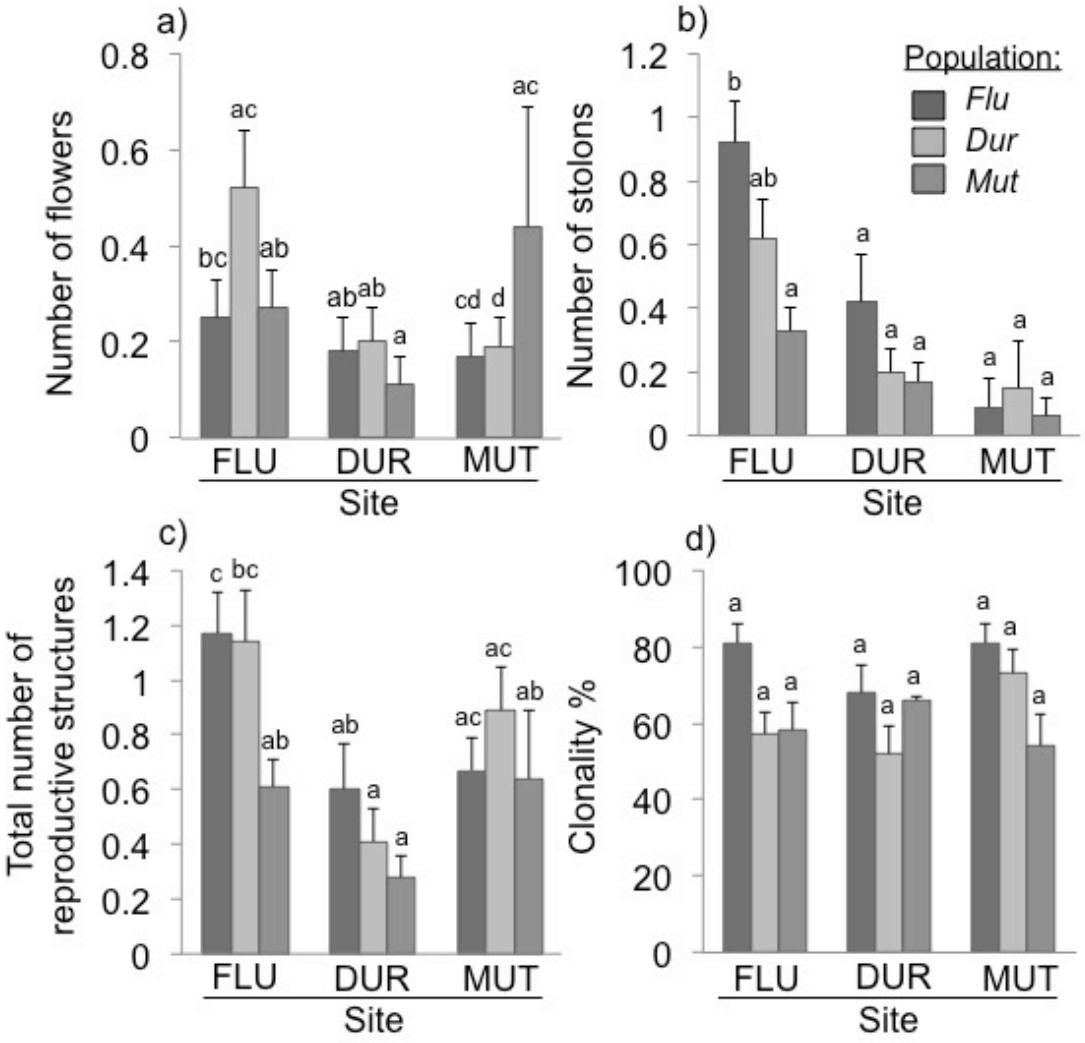

Fig. 2: Mean ± SE of reproduction-related traits: number of flowers (a), number of stolons (b), the total number of reproductive meristems (c), and the clonality (d) in *Geum reptans* populations (*Flu*, *Dur*, *Mut*) transplanted across three sites (Flüelapass: FLU, Dürrboden: DUR, Muttgletscher: MUT). Letters illustrate multiple contrasts (post-hoc Tukey HSD test) between site × population transplant combinations.

Leaf morphology

No significant interactions between site and population were detected for any of the traits related to leaf morphology (Table 3), indicating that populations did not differ in leaf morphology across transplant sites. However, highly significant site and/or population effects were detected for the leaf shape, leaf dissection and SLA, and leaf dissection also differed across genets (Table 3).

Site and population effects in the leaf shape were particularly pronounced for population *Mut* grown at the FLU site, which had a higher leaf shape (i.e. smaller leaf width for a constant leaf length) than when grown at the DUR or MUT site (Fig. 3a), and in contrast to the two other populations at the FLU site (Fig 3a). Similarly, site and population effects for leaf dissection were pronounced for population *Flu*, which had a higher leaf dissection ratio when grown at its site of origin (FLU) relative to when grown at the DUR site, and relative to population *Mut* when grown together at the FLU site (Fig. 3b). For SLA, the site effect was clearly visible when looking at population *Dur*, which displayed a significantly lower SLA when grown at its site of origin (DUR), relative to when grown at the FLU site (Fig. 3c).

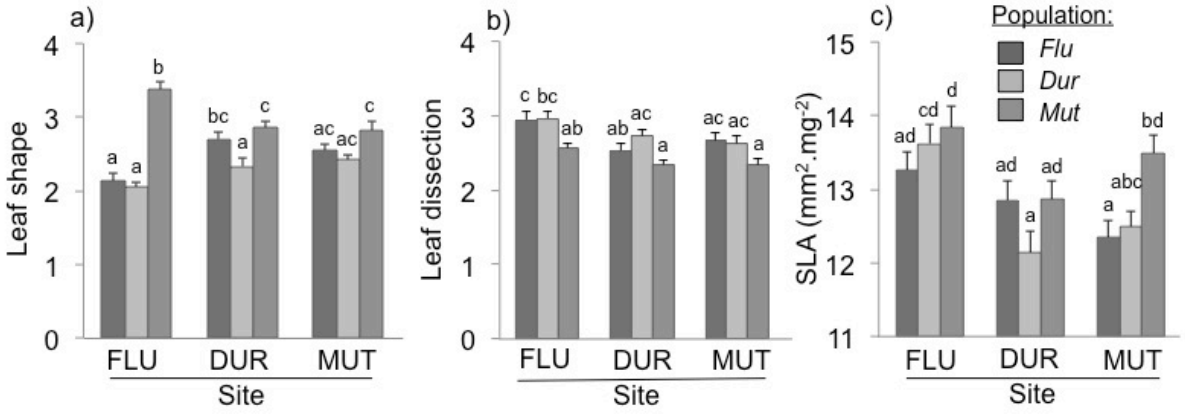

Fig. 3: Mean ± SE of leaf morphology traits: leaf shape (a), leaf dissection (b) and specific leaf area (c) in *Geum reptans* populations (*Flu*, *Dur*, *Mut*) transplanted across three sites (Flüelapass: FLU, Dürrboden: DUR, Muttgletscher: MUT). Letters illustrate multiple contrasts (post-hoc Tukey HSD test) between site × population transplant combinations.

Partitioning of genetic and environmental effects

For the growth-related traits, such as the aboveground dry mass and the number of leaves, environmental site effects explained about half of the trait variability (44.0% and 43.5%, respectively; Table 4). However, genetic effects at the level of the population or of the genets the remaining portion of the variance in these traits (Table 4).

For the reproduction-related traits, genetic population or genet effects explained the main part of trait variation, but environmental site effects also explained roughly a quarter of the variability in the number of stolons and total reproductive meristems (Table 4). For the number of flowers and clonality, none of the variation resulted solely from environmental effects, but was mainly explained by genet effects (Table 4).

Finally, for two of the three studied traits indicative of leaf morphology, environmental effects and genetic effects determined trait

variations at a similar proportion. Environmental site effects explained 50.1% of variation in leaf shape, and 37.3% in SLA. However, the variation in leaf dissection was mostly determined by genetic effects (27.6% population, 49.9% genet; Table 4).

Site-specific selection on trait values

Traits indicative of leaf morphology were found to be highly variable among transplant sites (Tables 3, 4). Thus, a selection analysis was performed to identify if selection for specific trait values occurred within experimental field sites. Most selection gradients calculated for SLA, leaf dissection and leaf shape at each site were found to be non-significant, suggesting no correlation between leaf morphology and individual fitness measured as total number of reproductive meristems (Table 5). Only the leaf shape was under direct linear selection at the MUT site where plants with a smaller ratio (i.e. wider leaves for constant leaf length) had a marginally higher fitness (non-significant after Bonferroni correction: β = -0.45, p = 0.12; Table 5).

Table 4: Variance components (%) of genetic effects (Population and Genets), environmentaleffects (Site) and interactive genotype × environment effects (Site × Pop) extracted for all fitness-related traits (growth and reproduction) and leaf morphology traits from three *Geum reptans* populations transplanted across three sites in the Central Swiss Alps.

	Site	Population	Site x pop	Genets
Growth-related traits				
Aboveground dry mass	43.99	22.98	3.18	29.84
Number of leaves	43.54	34.60	0.085	21.75
Reproduction-related traits				
Number of flowers	0	0	9.1	90.9
Number of stolons	26.63	36.13	9.27	27.97
Total reproductive meristems	23.68	9.46	1.50	65.36
Clonality	0	31.62	0	68.38
Leaf morphology				
Leaf shape	50.11	32.76	0	17.13
Leaf dissection	22.45	27.63	0	49.92
SLA	37.31	11.92	10.15	40.62

Table 5: Standardized linear selection gradients (β) and their level of significance (p-value) estimated as the multiple regression coefficients of relative fitness (i.e. total number of reproductive meristems) on standardized mean trait values at each field site. The p-value in italics was significant before Bonferroni correction (at $\alpha = 0.05$; p-values multiplied by 3 for correction), and non-significant p-values were truncated at 1 if >1 after correction.

	Flüelapass (FLU)		Dürrboden (DUR)		Muttgletscher (MUT)	
	β	p	β	p	β	p
Leaf shape	-0.151	1	-0.173	0.39	-0.45	*0.12*
Leaf dissection	-0.148	1	-0.031	1	-0.083	1
SLA	0.359	1	0.011	1	-0.048	1

Molecular differentiation

No identical multilocus genotypes (clonal offspring) were found across the 60 analyzed plants. We detected a mean number of alleles per population and locus of 7.18 ± 0.49, with a range of 3-11 alleles per locus. The mean genetic diversity (estimated as the unbiased expected heterozygosity) across all studied populations and loci was H_e = 0.72 ± 0.02. The genetic diversity within populations ranged from 0.69-0.74 and did not significantly differ among populations. Low inbreeding was revealed by F_{IS} = 0.16 ± 0.08 across all populations and loci. AMOVA revealed that 11% of molecular variance was found among populations (p = 0.001; Table 6), and 89% within populations (Table 6). Population pairwise F_{ST} values suggest that little molecular differentiation resided between the two close populations near Davos (*Flu* and *Dur*; F_{ST} = 0.016). However, higher F_{ST} values were found when comparing populations *Flu* and *Dur* to *Mut*, the more distant population at Muttgletscher (F_{ST} = 0.068 and F_{ST} = 0.073, respectively), suggesting higher molecular differentiation among distant populations.

Table 6: AMOVA results showing the molecular variance among and within populations.

Source	df	SS	MS	Est. Var.	%	*p*
Among populations	2	56.70	28.35	1.01	11	0.001
Within populations	57	460.10	8.07	8.07	89	-
Total	59	516.80	-	9.08	100	-

Discussion

Molecular differentiation and gene flow among populations

High genetic diversity was found within the three populations (H_e = 0.72). Our sampling method was designed in order to avoid picking the same genetic individual twice, by implementing at least 5 m distance between sampled individuals. This method was apparently successful since no identical multilocus genotypes were found, which also suggests that clonal ramets of *G. reptans* establish predominantly at close proximity to their mother plants (Pluess and Stöcklin, 2004, Hamann et al., 2014). Furthermore, this result suggests that the clonality of *G. reptans* did not cause a loss of genotypic diversity within populations, and is in line with previous findings reported in Pluess and Stöcklin (2004), and ultimately corroborates the consensus that populations of clonal species are often as genetically diverse as populations of non-clonal plants (Ellstrand and Elam, 1993, Widen et al., 1994). While sexual reproduction probably plays an important role for recruitment during founding stages of a population after glacier retreat (Cannone et al., 2008) and for the preservation of genetic diversity (Weppler et al., 2006), the long lifespan of clonal ramets and potential immortality of genets undoubtedly contributes to the maintenance of genotypic diversity in *G. reptans* (de Witte et al., 2011).

Molecular differentiation was substantial among populations (11%; Table 6). However, pairwise population F_{ST} comparisons revealed that molecular differentiation was low between the two populations growing at close proximity near Davos (*Flu* and *Dur*), yet both these populations differed strongly from the population *Mut* growing at a larger geographical distance at the MUT site. This

suggests that gene flow is maintained over distances of c. 5 km, despite the fact that these two populations are located in neighbouring valleys, which is nonetheless in accordance with a prior studies on pollen and seed dispersal distances (Pluess and Stöcklin, 2004, Tackenberg and Stöcklin, 2008).

Little evidence for local adaptation

No differences were found in growth or reproductive traits between populations transplanted back to their home site or to foreign sites (i.e. sympatric vs. allopatric contrast). The frequency of individual survival and reproduction also did not differ across the sympatric vs. allopatric contrast, and only the frequency of flowering was lower in far-allopatric transplant combinations. Hence, our results suggest only little evidence for local adaptation in the studied *G. reptans* populations from the Central Swiss Alps, even when separated by relatively large geographic distances, where gene flow is probably restricted.

Evidence for local adaptation has been found in a number of alpine plant populations (Gonzalo-Turpin and Hazard, 2009, Fischer et al., 2011, Giménez-Benavides et al., 2011, Hamann et al., 2016), however, an extensive meta-analysis and another recent study suggest that local adaptation may be less common than frequently assumed (Leimu and Fischer, 2008, Hirst et al., 2016). Extensive gene flow among populations has been recognized as a main hindrance for local adaptation (Kawecki and Ebert, 2004). Given the low level of molecular differentiation found in our study among populations at close proximity, this could potentially explain the lack of phenotypic differentiation between the two populations growing at the sites near Davos (FLU and DUR), but fails to do so for the more distant population at the MUT site. Nevertheless, the two nearby populations are c. 5 km apart, making genetic swamping very unlikely.

While it is possible that local adaptation may take more time than allowed in our experiment to express depending on plant longevity (Bennington et al., 2012, Hirst et al., 2016), the most likely explanation for the lack of local adaptation in our study is related to the narrow habitat niche of *G. reptans*. This species grows at high elevation, typically in glacier forelands, close to the glacier snout, and in moist scree fields (Aeschimann et al., 2004). Consequently, it is likely that environmental conditions are very similar in these habitats, regardless of geographic distance, which may explain the lack of intraspecific differentiation (Cannone et al., 2008, Cheplick, 2015). Indeed, differences in elevation, temperature, precipitation and exposition recorded in our study (Table 1) might not be substantial enough to lead to divergent selection. Supporting this interpretation, the selection analysis for mean leaf traits at different sites showed only little direct linear selection on these traits (Table 5), corroborating the fact that there was no divergent selection across the studied sites. Since only three populations from the Central Swiss Alps were studied here, it is important to note that adaptive genetic differentiation may in fact be found across larger geographic ranges, and such genetic differentiation may well be in line with this species' glacial history and postglacial recolonization (Frei et al., 2012).

An alternative, not mutually exclusive, explanation for the lack of local adaptation in our study could be that highly plastic phenotypic responses to local environmental conditions may overcome the need for genetic differentiation among populations, especially in perennial herbs (Antonovics and Primack, 1982, Bazzaz, 1996, Cheplick, 2015, Hirst et al., 2016). Indeed, our study

revealed that *G. reptans* had a great capacity to respond plastically to environmental conditions (Tables 2 and 3), which can represent a means to maximize plant performance in heterogeneous environments (Alpert and Simms, 2002, Stöcklin et al., 2009, Nicotra et al., 2010). This may be especially true when considering the relatively narrow range of environmental conditions in the glacier forelands studied here, which may be within the limits that plants can adjust to by means of plastic responses (Alpert and Simms, 2002). However, future studies should investigate the adaptive value of trait plasticity in contrast to genetic differentiation in more detail and across the entire geographical and ecological range of *G. reptans*.

Phenotypic differentiation: environmental vs. genetic effects

While populations transplanted back to their home sites did not outperform populations transplanted to foreign sites, our experiment revealed certain differences in site characteristics (Tables 2 and 3). Especially plants grown at the MUT site had a higher aboveground dry mass and produced a greater number of leaves compared to when grown at the other sites (Fig. 1). Variations in these traits were generally strongly driven by environmental conditions (Table 4). While we mentioned earlier that environmental conditions in glacier forelands are relatively similar, they can differ in the time lapse since glacier retreat and hence in their successional stage (Cannone et al., 2008). Indeed, the MUT site, where glacier retreat started in the mid 1990's, is at an earlier successional stage than the two sites near Davos, where glacier retreat started in the late 19th century (Schweizerisches-Gletschermessnetz, 2015). Hence, this site is still at an early-successional stage, and might allow for better growth of pioneer and early-successional species, such as *G. reptans*, relative to sites at a later-successional stage where interspecific competition increases (Cannone et al., 2008). Similarly, the number of stolons and of total reproductive meristems produced by individuals was lower at the DUR site (Fig. 3), where higher competition might have hindered optimal reproduction.

Leaf morphology also varied greatly in response to environmental conditions at transplant sites. Variation in SLA equally reflected environmental and genetic differences among genets, and variation in the leaf shape predominantly resulted from plastic responses to environmental site conditions (Table 4). All these traits can help optimize light capture and gas exchange (Wright et al., 2004, Poorter et al., 2009), and may have positive repercussions on plant fitness if rapidly adjustable across diverse environments.

While genetic population and genet effects explained a large part of phenotypic variation in reproductive traits (Table 4), the reproductive output of individuals also varied between transplantation sites (i.e. plasticity). The low frequency of flowering individuals, and the high relative proportion of reproduction via clonal ramets (Fig. 2d) were probably related to the young age and small size of our experimental plants as found in prior studies (Pluess and Stöcklin, 2005, Weppler et al., 2006). Pluess and Stöcklin (2005) also revealed a great size-dependent plasticity in the reproductive strategy of *G. reptans*, which ensures population persistence and reproduction across a range of habitat conditions, and corroborates our hypothesis that phenotypic plasticity might prevail over genetic differentiation in *G. reptans* growing in glacier forelands in the Swiss Alps.

Conclusion

Our study revealed only little evidence for local adaptation of *G. reptans* populations growing on the studied glacier forelands in the Central Swiss Alps, even though extensive molecular differentiation was found between the far-away populations. We suggest that the niche of this species is relatively narrow, and restricted to similar environmental conditions in glacier forelands and moist screes. Moreover, both growth- and reproduction-related traits, as well as leaf traits exhibited strong phenotypic plasticity, which may overcome the need to adapt by means of genetic differentiation. Since only a limited number of populations were studied here, we cautiously advocate that selection could have led to the evolution of phenotypic plasticity rather than genetic differentiation, and encourage future studies to investigate the adaptive value of phenotypic plasticity across the natural range of this species.

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Declaration of authorship and conflicts of interest

Data and findings presented in this manuscript have not been published and are not under consideration for publication elsewhere. All the authors have approved this submission and all persons entitled to authorship have been named. The authors have no conflict of interest to declare.

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Chapter 7

Spatial patterns of local adaptation in two common herbs from the central European Alps

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Abstract

- Spatially variable selection is considered to result in local adaptation. Yet the generality of local adaptation of populations remains debated, and we know little about the spatial patterns of local adaptation.
- We conducted reciprocal transplantations among six populations each of two common and well-studied herbaceous plants, *Anthyllis vulneraria* and *Arabis alpina*. We measured aboveground biomass, reproductive allocation and flowering propensity to test for local adaptation at two spatial scales: within and between the Eastern and Western Swiss Alps. Additionally, populations were genotyped using microsatellite markers to assess neutral differentiation and historic inbreeding.
- Microsatellite analyses indicated neutral population differentiation according to spatial scale in both species, as well as mixed mating in *Anthyllis vulneraria* and strong inbreeding in *Arabis alpina*. The spatial scale was also mirrored in fitness variation of transplanted *Anthyllis vulneraria*: fitness decreased with geographic distance between population origin and transplant site. In *Arabis alpina*, reproductive biomass was lowered only in near away transplantations, but not in far away transplantations.
- The findings suggest that habitat heterogeneity across the alpine landscape can drive local adaptation although results on *Arabis alpina* remain inconclusive. Selection-driven differentiation appears to increase with geographic distance in the outcrossed *Anthyllis vulneraria*.

Keywords: reciprocal transplantation; spatial scale, alpine plants; *Anthyllis vulneraria*; *Arabis alpina*, microsatellites, inbreeding, outcrossing.

Introduction

Despite the long history of research on local adaptation of plant populations (Clausen et al., 1941), there is still debate over its ubiquity (Leimu and Fischer, 2008, Hereford, 2009) and best definition (Kawecki and Ebert, 2004, Blanquart et al., 2013). Ideally, proof of local adaptation should demonstrate an increase in individual fitness through evolution from the ancestral state towards the descendant state (Lande and Arnold, 1983, Travisano et al., 1995). The ancestral state, however, is usually not conserved through time (but see Franks et al., 2007, Franks and Weis, 2008), making this comparison impossible. The reciprocal-transplant design has therefore become the standard experimental design for testing local adaptation of populations (Kawecki and Ebert, 2004, Blanquart et al., 2013).

Two criteria have traditionally been applied to test for local adaptation in reciprocal transplant studies: the *local vs. foreign criterion* and the *home vs. away criterion* (Kawecki and Ebert, 2004). The *local vs. foreign criterion* compares the fitness of the local population to foreign populations at each transplantation site, while the *home vs. away criterion* compares the fitness of each population at their home sites to their fitness at away sites. Both criteria are not free of confounding effects: the former is confounded by the intrinsic vigour of the populations and the latter by the fertility of the sites. One frequently finds differences among populations in their intrinsic vigour that need not be related to local adaptation (Leimu and Fischer, 2008, Hirst et al., 2016), and likewise, the quality of sites can vary greatly across the landscape. Therefore, these confounding effects are serious problems in the analysis of reciprocal transplant experiments. To cope with these confounding factors, a novel criterion has recently been introduced, called the *sympatric vs. allopatric criterion* sensu Blanquart et al. (2013). It compares the average fitness in naturally occurring population by site combinations (*sympatric*) to the average fitness in experimentally created population by site combinations (*allopatric*) across a preferably large number of populations. Before the criterion is assessed, effects of population and site quality are statistically removed, and therefore, effects unrelated to local adaptation do not confound the *sympatric vs. allopatric criterion*.

It is of particular interest to test local adaptation across multiple ecological scales (e.g. habitat types or geographical scales) to resolve some of the disagreement over the ubiquity and conditions under which local adaptation evolves (Peterson *et al.*, 2016). The *sympatric vs. allopatric criterion* is ideal to incorporate multiple ecological scales because it can be divided into multiple levels with little loss of statistical power (Blanquart et al., 2013), for example according to the geographic distance between a transplantation site and the site of origin of a population (e.g. *sympatric*, *near allopatric*, *far allopatric*).

The evolution of local adaptation among plant populations is expected when populations of a species experience consistent divergent selection, are sufficiently genetically isolated, and have high genetic variation. Accordingly, the signature of local adaptation should be strong when comparing plant populations at great geographic or environmental distance, and weak at small distances. Few studies have set out to explicitly test such hypotheses at multiple ecological scales simultaneously. Evidence is correspondingly rare, but mostly supportive (Sambatti and Rice, 2006, Hereford and Winn, 2008, Anderson et al.,

2015, Peterson et al., 2016). Galloway and Fenster (2000) have found evidence for local adaptation of an annual legume at distances greater than 1'000 km, but not at closer distances. Likewise, (Torang et al., 2015) have found strong local adaptation in the arctic-alpine *Arabis alpina* at distances of 3'000 km, but not at distances smaller than 600 km. The only study to date that has made use of the *sympatric vs. allopatric criterion* for testing local adaptation at multiple spatial scales is by Hamann et al. (2016), who have found that a common alpine fodder grass shows local adaptation at the regional scale (>200 km), along with some evidence for adaptive differentiation at the within-region scale (>20 km).

Mountain plants are particularly interesting for the study of local adaptation because they frequently face diverse habitats across their range even at similar elevation (Körner, 2003). While studies in mountain systems usually find considerable phenotypic differentiation among plant populations (Pluess and Stöcklin, 2004, Byars et al., 2007, Giménez-Benavides et al., 2007, Gonzalo-Turpin and Hazard, 2009, Stöcklin et al., 2009, Frei et al., 2014), there is mixed evidence for the prevalence of local adaptation at high elevations (Galen and Stanton, 1991, Angert and Schemske, 2005, Geber and Eckhart, 2005, Byars et al., 2007, Gonzalo-Turpin and Hazard, 2009, Sedlacek et al., 2015, Hirst et al., 2016). Furthermore, we lack studies that focus on local adaptation of mountain plants unrelated to elevational gradients, i.e. local adaptation of plant populations at similar elevation (Hirst et al., 2016).

In the current study, we performed reciprocal transplantations across two spatial scales within the Swiss Alps (within and between the Eastern and Western Swiss Alps) using two alpine species, *Anthyllis vulneraria* L. and *Arabis alpina* L. These species were chosen because they are widely distributed and well-studied herbaceous plants of the European Alps. Each of six populations per species was transplanted to its site of origin, to another site in the same region, and to a site in the other region. We measured mortality, aboveground biomass, and flowering propensity as fitness proxies, and tested for local adaptation using the *sympatric vs. allopatric criterion*. We also investigated genetic population structure, genetic diversity, and inbreeding levels using microsatellite markers. The following questions were addressed: (1) Is there evidence for local adaptation in alpine *Anthyllis vulneraria* and *Arabis alpina*? (2) Does the geographic distance between transplant sites explain fitness variation, i.e. is the occurrence and strength of local adaptation related to the spatial scale? (3) Do the experimental populations show neutral genetic differentiation, and is this differentiation in line with their geographic distribution?

Materials and Methods

Study species

Anthyllis vulneraria L. sensu lato (s.l.; Fabaceae) is a clade of self-compatible short-lived perennial rosette plants common throughout Europe. It grows preferably on calcareous grassland and scree up to around 3'000 m above sea level (m a.s.l) (Conert et al., 1995). Plants grow to a height of ca. 15-45 cm. Each plant comprises a variable number of shoots, of which each bears 2-6 inflorescences. Each inflorescence comprises a number of 7-19mm long white to yellow, sometimes claret to red flowers arranged in a capitulum (Conert et al., 1995, Navarro, 1999a). Selfed and geitonogamous offspring may be produced due to the spatial co-

location of self-pollen and stigma and the asynchronous flower ripening across capitulae and shoots. Populations of *Anthyllis vulneraria* may be exclusively selfing (Couderc, 1971) or may be protandrous to a degree where selfing is effectively prevented (Navarro, 1999b). *Anthyllis vulneraria* s. l. is a particularly polymorphic taxon with unclear infraspecific classification (Nanni et al 2004; Köster et al 2008). We have assigned the alpine populations studied here to *Anthyllis vulneraria* ssp. *alpestris* (Schult.) and to *Anthyllis vulneraria* ssp. *valesiaca* (Beck) (Lauber and Wagner, 2001).

Arabis alpina L. (Brassicaceae) is a perennial rosette plant (Conert et al., 1995, Karl and Koch, 2013). *Arabis alpina* has a wide distribution range from the high mountains of northern Africa over the Pyrenees and the European Alps to the Near East, and across the whole arctic region. *Arabis alpina* is a pioneer plant and grows near glacier snouts and on screes up to around 3'200 m a.s.l., but can also be found at lower elevations down to 400 m a.s.l. It occurs commonly on disturbed and mildly moist sites with calcareous and alkaline bedrock (Conert et al., 1995, Koch et al., 2006). *Arabis alpina* grows 6-40 cm tall. Vegetative shoots are short and horizontally crawling with leaf rosettes at the tip of the shoots, while reproductive shoots are upright. Flowers produce nectar and are arranged as raceme. *Arabis alpina* populations from the central and western Alps are highly inbred due to a non-functional self-incompatibility system (Buehler et al., 2012) resulting in frequent selfing along with bi-parental inbreeding.

Molecular genotyping

20 individuals per study population of *Anthyllis vulneraria* were scored for amplified fragments at 9 microsatellite loci, and 15 individuals per study population of *Arabis alpina* at 10 loci. Six study populations per species were used (detailed below). Leaf samples for DNA extraction were taken from experimental plants. Each sample was taken from one randomly chosen offspring of a different maternal plant each, and stored in paper bags in silica gel. We used Spreadex® gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) to separate PCR amplicons with size differences as small as 2bp. Gels were stained with ethidium-bromide and scored by hand comparing against ELCHROM's M3 ladder. A detailed description of the microsatellite analysis and *Anthyllis vulneraria* loci can be found elsewhere (Kesselring et al., 2013). New primers were designed for *Arabis alpina* based on published GenBank sequences of 10 loci described by Buehler et al. (2011) to achieve an amplicon length of 90-150 bp, which is suitable for Spreadex electrophoresis (Kesselring et al., 2013). PCR details and primer sequences are reported in the supplementary materials of the primer note cited above. Error rate in electrophoretic genotyping of *Arabis alpina* was estimated with a repetition analysis, starting from DNA extraction of 11 of the 105 individuals (9.5% of the entire sample size). 216 signals of amplified DNA were found in the first run for these 11 individuals across all 10 loci. In the repetition analysis, 212 of the 216 alleles were identically re-scored, equalling an error rate of 1.8%. Error of *Anthyllis vulneraria* was estimated at 2.5% (Kesselring et al., 2013). Null alleles were suggested for all loci in most populations of *Anthyllis vulneraria* by the software FreeNA (Chapuis and Estoup, 2007). However, F_{ST} values adjusted for null alleles were nearly identical for all but one locus and only this locus showed homozygote null alleles (blank

lanes on the gel). Visual inspection of electrophoresis gels and the biology of the species furthermore suggest that the observed heterozygote deficiencies at the other loci are not due to artefacts. Therefore, we removed only the questionable locus from analyses and assumed the rest of the data to be free of artefacts. *Arabis alpina* showed only two blank lanes out of 900 making it highly unlikely that null-alleles exist in the studied samples, especially given the fact that *Arabis alpina* is highly inbred.

Reciprocal transplantations

For both species, three populations from each of two regions, namely Davos and Zermatt were used for transplantations (Table 1). The distance between regions roughly equals 180 km, and distances between populations within regions range from 2 to 18 km. Juvenile offspring of each population were transplanted to their *home* site, to an *away* site in the same region, and to a *far away* site in the other region. It follows that each site received offsprings from its *local* population, from a foreign population of the same region (*near foreign*), and from a foreign population of the other region (*far foreign*). Thus, a total of 18 transplantations were performed per species. Sensu Blanquart et al (2013), the combination of a site with its local population is referred to as *sympatric*, one of a site with a foreign population as *allopatric* (again, note that the terms sympatric and allopatric do not have the same meaning as in classical population biology). In this study, we further specify the combination of a site with a population from the same region as *near allopatric*, and a combination of a site with a foreign population from the other region as *far allopatric*. Populations and sites for *near allopatric* and *far allopatric* transplantations were randomly matched. We preferred an unbalanced reciprocal design instead of transplanting all populations to all sites, as a fully factorial design has been shown to not make optimal use of resources in terms of statistical power (Blanquart et al., 2013).

For *Anthyllis vulneraria,* open-pollinated seeds were collected from 45 different maternal plants in the second week of August 2012 and stored in separate paper bags to subsequently trace family membership. One week later, between 15-Aug-2012 and 17-Aug-2012, seeds were scarified and placed on wet filter paper in Petri dishes for germination in the glasshouse. On 21-Aug-2012, three seedlings per maternal family were potted into 54-pot trays filled with low nutrient soil (Anzuchterde, Ökohum GmbH, Herrenhof, Switzerland). Final transplantation to field sites was performed on 17 and 18-Sep-2012 in Davos and on 25 and 26-Sep-2012 in Zermatt. Of the three individuals per maternal family, one was transplanted to its *home* site, one to the *away* site, and one to the *far away* site. However, family membership was ultimately ignored, because uneven mortality led to an unequal genetic make-up of transplanted populations. Plants were transplanted into the local soil at the field sites of the native population and each plant was watered with 200 ml of water to facilitate establishment. Plants were transplanted in rows of 10 individuals, alternating between *local*, *near foreign*, and *far foreign* individuals, and spacing individuals at 20 cm distance from each other. Each site received 135 individuals that were arranged in 14 rows. A total of 810 individuals were reciprocally transplanted for *Anthyllis vulneraria*.

Table 1: Elevations (m.a.s.l) and coordinates (Swiss grid CH1903) of the 12 populations from two regions of *Anthyllis vulneraria* and *Arabis alpina* used for reciprocal transplantations.

	Anthyllis vulneraria			*Arabis alpina*		
	Population	Coordinates	Elevation	Population	Coordinates	Elevation
Davos (Eastern Swiss Alps)	Schiahorn	780513.38/ 187874.76	2650	Schiahorn	780513.385/ 187874.756	2650
	Casanna	782301.54/ 192247.99	2320	Casanna	782301.543/ 192247.969	2320
	Monstein	779685.63/ 173389.10	2010	Weissfluhjoch	780324.165/ 189706.004	2700
Zermatt (Western Swiss Alps)	Findelwald	626828.96/ 95475.764	2170	Blauherd	627165.547/ 96339.072	2580
	Findelgletscher	629173.61/ 95175.270	2490	Findelgletscher	629173.611/ 95175.270	2490
	Stafelalp	619094.30/ 94427.436	2280	Trockener Steg	621622.757/ 90793.274	2880

Towards the end of the first growing season in the field (September 2013), we assessed survival at all transplantation sites. Earlier, in the first week of July 2013, we had assessed survival at one site in Davos (Monstein) and one site in Zermatt (Findelgletscher). Thus by comparing September survival against July survival, we could assess mortality of established individuals during the first summer at these two sites (n = 165). To assess mortality during the second winter, we compared data from September 2013 against survival at the time of harvest in 2014.

After two full growing seasons in the field (August 2014), we monitored whether individuals had flowered or not and harvested aboveground biomass. Biomass was divided into reproductive and vegetative biomass and dried in the oven at 80 °C for 48 h. For final analysis of biomass in *Anthyllis vulneraria* 304 plants remained.

For *Arabis alpina*, open-pollinated seeds were collected from 45 maternal plants during September and October 2012. Seeds were kept at room temperature for one week and then stored at 4 °C. Three seeds per maternal line were sown on 27-Mar-2013 onto small trays filled with low nutrient soil and topped with a thin layer of soil (Anzuchterde, Ökohum GmbH, Herrenhof, Switzerland). Seeds were subsequently cold-stratified at 4 °C for 4 days to improve germination rate. Between 18-Apr-2013 and 29-May-2013 seedlings were transferred to 54-pot multitrays containing the same soil. Plants were transplanted to field sites between 03-Jul-2013 and 26-Jul-2013. Transplantations were performed in the same fashion as with *Anthyllis vulneraria*. A total of 810 individuals were transplanted.

Since *Arabis alpina* individuals grew in the field for only one growing season, we did not assess mortality because we would not be able to separate site effects from transplantation effects. However, at the end of the growing season we assessed propensity of flowering and harvested aboveground biomass between 31-Jul-2014

and 05-Aug-2014. Reproductive and vegetative biomass was separated and dried in the oven at 80 °C for 48 h.

Statistical analysis

We used analysis of molecular variance (AMOVA) as implemented in GenAlEx to partition genetic variation at microsatellite loci into components according to the sources region, population within region, individual within population, and within individuals. AMOVA also estimates Wright's fixation indices (*F*) corresponding to structuring at the same hierarchical levels. We further report genetic diversity at microsatellite loci in terms of expected heterozygosity H_e and allelic richness.

We used linear mixed effects models for the analysis of biomass traits based on the *sympatric vs. allopatric* definition of local adaptation (Blanquart et al 2013). To this end, we specified models in the lmerTest package (Kuznetsova 2013) for R (R Development Core Team 2008), which included a factor for site, population and a factor describing whether a combination of site and population was *sympatric*, *near allopatric*, or *far allopatric*. The factors site, population and *sympatric vs. allopatric* were tested for their effects on total aboveground biomass and reproductive allocation. Separate models were specified for each response variable and species. Site and population, were specified as random effects, while *sympatric vs. allopatric* was a fixed effect. lmerTest is a package of convenience functions for lmer objects of the lme4 package (Bates, 2014) that allow F-tests for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and comparison. We used Type 3 sums of squares and Satterthwaite approximations for degrees of freedom. We report *P*-values, mean squares, and χ^2 values that correspond to those from the model comparison (i.e. likelihood-ratio tests) using the step function in lmerTest.

Significant site or population terms indicate differences in intrinsic habitat quality or population quality, respectively. A significant *sympatric vs. allopatric* factor indicates that populations perform on average better when transplanted to either one of the *home*-, *near away*- or *far away sites*. If the *sympatric vs. allopatric* factor was significant, post-hoc pairwise comparisons were used to check if patterns of fitness variation conformed to local adaptation. A significantly positive difference between *sympatric* combinations of sites and populations and *near allopatric* combinations of sites and populations indicates local adaptation at the scale of populations within regions. A significantly positive difference between *sympatric* combinations of sites and populations and *far allopatric* combinations of sites and populations indicates local adaptation at the regional scale. We used differences of least squares means (dlsm) as output by the step function of lmerTest for post-hoc comparisons.

Flowering propensity and survival of *Anthyllis vulneraria* were analysed using generalized linear mixed-effects models of the lme4 package (Bates et al., 2015) for R (R Development Core Team, 2013) with a binomial distribution and the logit link function. Identical model specifications were used as for the mixed effects models above. Likelihood ratio tests were performed to assess significance levels of all factors. The glht function of the multcomp package (Hothorn et al., 2014) in R was used for post-hoc pairwise comparisons of the levels of the *sympatric vs. allopatric* factor.

Results

Molecular Analyses

In *Anthyllis vulneraria*, average numbers of alleles per locus and population ranged from (mean±SD) 5.80±0.56 to 8.00±1.05 and expected heterozygosities (H_e) ranged from 0.68±0.02 to 0.76±0.02 across populations. The microsatellite analyses revealed a positive mean inbreeding coefficient (F_{IS}) of 0.254 across populations of *Anthyllis vulneraria* used in this study, ranging from 0 to 0.414 across populations. AMOVA showed that small but significant amounts of molecular variation are explained by region (5%) and population structure within regions (4%; Table 2). Pairwise F_{ST}'s ranged from 0.014 to 0.084 within the Davos region, from 0.000 to 0.052 within the Zermatt region, and from 0.038 to 0.127 across regions. Only the populations Findelgletscher and Stafelalp were not significantly differentiated from each other (Table S1).

In *Arabis alpina*, average numbers of alleles per locus and population ranged from 2.60±0.34 to 3.80±0.44. Expected heterozygosities (H_e) ranged from 0.34±0.07 to 0.54±0.04 across populations. Our analyses confirmed the highly inbred nature of *Arabis alpina* with an average F_{IS} of 0.758 for the populations studied here (ranging from 0.459 – 0.975). Accordingly, AMOVA revealed that most of the molecular variability is between (55%) and not within individuals (17%; Table 2). Furthermore, regions and populations within regions explained relatively large amounts of variation with 11 % and 17 %, respectively. Pairwise F_{ST}'s ranged from 0.048 to 0.127 within the Davos region, from 0.101 to 0.257 within the Zermatt region, and from 0.155 to 0.282 across regions (Table S2).

Transplant experiments

Anthyllis vulneraria

Mortality after establishment was assessed for *Anthyllis vulneraria* in the first growing season (summer 2013) at one site in each region and at all sites during the second winter (2013/2014). During summer 2013, only a single individual died at the Findelgletscher site in the Western region, and seven individuals died at the Monstein site in the Eastern region. Mortality during the second winter in the field (2013/2014) was higher: out of 437 plants living at all six sites in September 2013, 122 (28%) died until the final harvest in September 2014. However, mortality during the 2013/2014 winter was not dependent on the *sympatric vs. allopatric factor* (results not shown).

In contrast, while the flowering propensity assessed at the end of the two growing seasons did not differ between populations or sites (results not shown) it differed significantly along the *sympatric vs. allopatric* contrast (Fig. 2; *sympatric vs. allopatric* factor: df = 2, $\chi^2 = 6.96$, $P = 0.031$). Sympatric combinations had 76 % flowering propensity, allopatric combinations 66 %, and far allopatric only 62%. Generally, the population of origin at each site had the highest flowering propensity, the only exception being the Stafelalp site. In all populations transplanted to the Stafelalp site, less than 50% of plants flowered due to the low overall growth of transplanted plants (see also biomass results).

Total aboveground biomass of *Anthyllis vulneraria* differed between populations and sites, and also along the *sympatric vs. allopatric* factor (Table 3). The average total aboveground biomass across the experiment was lower in *far allopatric* plants compared to *sympatric* and *near allopatric* transplant combinations (Fig. 1; dlsm: sym-far.allo=0.5, p<0.001; near.allo-far.allo=0.5, p<0.001).

Table 1: Elevations and coordinates (Swiss grid CH1903) of the 12 populations from two regions of *Anthyllis vulneraria* and *Arabis alpina* used for reciprocal transplantations

	Anthyllis vulneraria			***Arabis alpina***		
	population	coordinates	elevation	population	coordinates	elevation
Davos (Eastern Swiss Alps)	Schiahorn	780513.385 / 187874.756	2650	Schiahorn	780513.385 / 187874.756	2650
	Casanna	782301.543/ 192247.969	2320	Casanna	782301.543/ 192247.969	2320
	Monstein	779685.630 / 173389.160	2010	Weissfluhjoch	780324.165 / 189706.004	2700
Zermatt (Western Swiss Alps)	Findelwald	626828.986 / 95475.764	2170	Blauherd	627165.547 / 96339.072	2580
	Findelgletscher	629173.611 / 95175.270	2490	Findelgletscher	629173.611 / 95175.270	2490
	Stafelalp	619094.320 / 94427.436	2280	Trockener Steg	621622.757 / 90793.274	2880

Table 2: AMOVA table (analysis of molecular variance) of 12 populations from two regions of *Anthyllis vulneraria* and *Arabis alpina*. Degrees of freedom (df), sums of squares (SS), estimated variance (est. var.), percentage variance explained by each factor (%), and the magnitude of the fixation index at each hierarchical level (fixation index) are given. All fixation indices were significant at the *P*=0.001 level (**).

	Anthyllis vulneraria					*Arabis alpina*				
	df	SS	Est. var.	%	Fixation index	df	SS	Est. var	%	Fixation index
region	1	25.7	0.145	5	0.045 **	1	53.9	0.355	11	0.105 **
population	4	33.8	0.114	4	0.037 **	4	87.8	0.589	17	0.195 **
individual	114	422	0.751	23	0.081 **	84	359.7	1.847	55	0.279 **
within individual	120	264	2.2	69	0.254 **	90	53	0.589	17	0.758 **
total	239	744.7	3.21	100	0.315 **	179	554.4	3.38	100	0.826 **

Table 3: ANOVA table of mixed model analyses of total aboveground biomass and reproductive biomass for *Anthyllis vulneraria* and *Arabis alpina*. F-tests were used for fixed effects and likelihood ratio tests for random effects as implemented in the lmerTest package for R. lmerTest allows for variances of 0, resulting in NA's for F- and χ^2-values.

		Anthyllis vulneraria						***Arabis alpina***					
		Total biomass			Reproductive biomass			Total biomass			Reproductive biomass		
	df	MS[b]	F/$\chi^{2,a}$	p	MS[b]	F/$\chi^{2,a}$	p	MS[b]	F/$\chi^{2,a}$	p	MS[b]	F/$\chi^{2,a}$	p
site	4	NA	106.36	<0.001	NA	25.37	<0.001	NA	106.36	<0.001	NA	0.00	1.000
population	5	NA	10.95	<0.001	NA	2.92	0.088	NA	10.95	<0.001	NA	0.87	0.350
sympatric vs. allopatric	2	7.96	15.52	<0.001	0.43	8.81	<0.001	7.96	15.52	<0.001	0.14	4.44	0.015

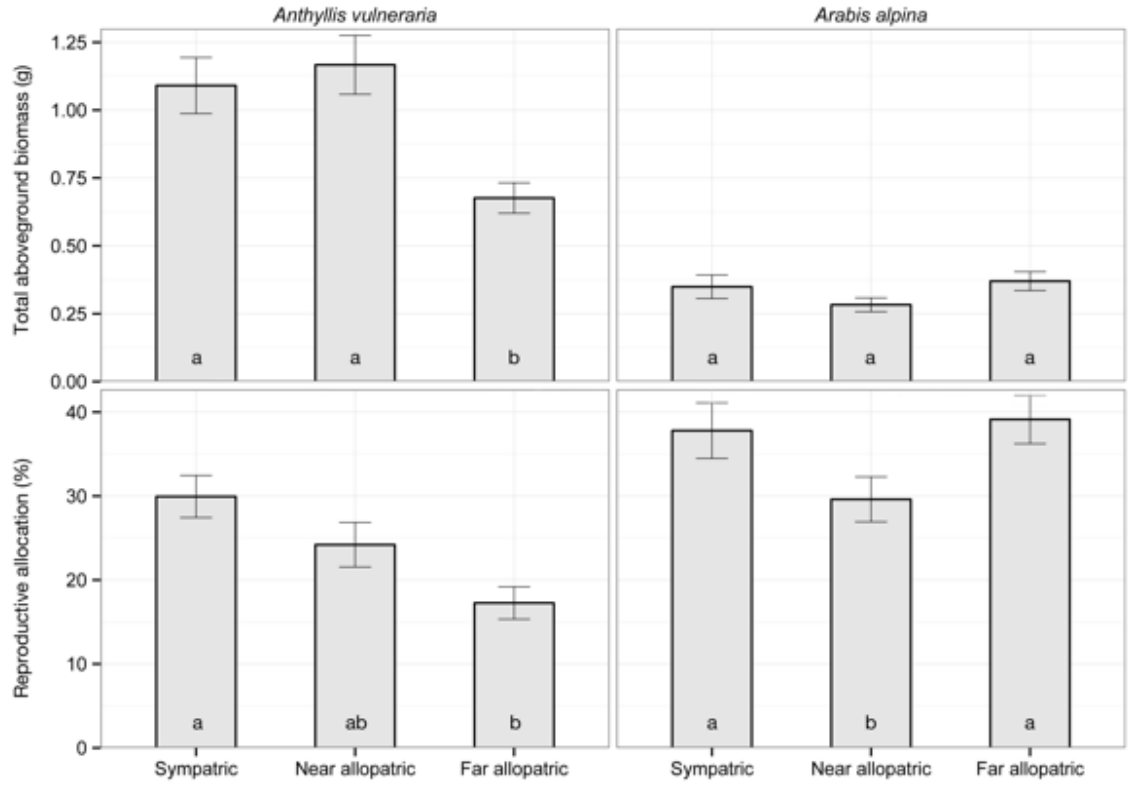

Fig. 1 Total aboveground biomass and allocation to reproductive biomass for *Anthyllis vulneraria* and *Arabis alpina* for *sympatric*, *near allopatric* and *far allopatric* transplant combinations. Error bars depict 1 standard error of the mean. Different small letters inside bars of each panel denote significantly different groups.

Near allopatric combinations of populations and habitat within the same region had equal total aboveground biomass production to *sympatric* transplants (Fig. 1; dlsm: sym-near.allo=0.0, p=0.9).

Allocation to reproductive biomass also differed among sites and among populations (Table 3; population p=0.002; site p=<0.001) Moreover, a highly significant *sympatric vs. allopatric* effect was found for this trait (p=<0.001). Reproductive biomass of *Anthyllis vulneraria* tended to decrease with increasing distance between transplantation site and population origin (Fig. 1). *Sympatric* transplantations yielded the highest reproductive biomass, *near allopatric* intermediate, and *far allopatric* transplantations lowest biomass (dlsm: sym-near.allo=0.1, p=0.038; sym-far.allo=0.3, p<0.0001, near.allo-far.allo=0.2, p=0.007).

Arabis alpina

Early timing of transplantations and late spring frost caused severe mortality at transplantation and transplants from two sites were completely lost. In total, only 200 individuals survived and were available for final analyses. Out of the 200 surviving *Arabis alpina* plants at four sites, all but 28 flowered at the time of harvest. Of these 28 non-flowering plants, 11 were in sympatric transplant combinations with their habitat, 5 were in *near allopatric*, and 12 were in *far allopatric* ones. None of the factors site, population or *sympatric vs. allopatric* explained significant variation (results not shown).

Total aboveground biomass of *Arabis alpina* was not differentiated among populations or sites. The *sympatric vs. allopatric* factor was also not significant for total biomass (Table 3; Fig. 1).

Similarly, there were no significant differences among sites or among populations in reproductive biomass (Table 3). However, the *sympatric vs. allopatric* factor was significant for reproductive biomass in the *Arabis alpina* experiment, with *near allopatric* combinations having lower biomass than *sympatric* and *far allopatric* population by habitat combinations (Table 3; dlsm: sym-near.allo= 0.1, p=0.02; near.allo-far.allo=<0.1, p=0.03; Fig. 1). Cross-regional transplant combinations (*far allopatric*) were not significantly different from *sympatric* combinations (Fig. 1; dlsm: sym-far.allo=0.00, p=0.78).

Discussion

Determining the structure of fitness variation and the scale of adaptive differentiation in widespread plant species is important for understanding the ecology of these species and their evolutionary potential. In the current study, we transplanted six populations of each of two widespread alpine species across two spatial scales and tested for local adaptation. We found a signature of local adapation in *Anthyllis vulneraria*, for which the reproductive biomass and flowering propensity decreased with increasing distance of sites of origin. However, in *Arabis alpina,* no conclusive evidence for local adaptation was found.

Differentiation at microsatellite loci of both species showed stronger differentiation across than within regions. The additional genetic isolation between regions should therefore favor regional differentiation over local differentiation. Populations of *Anthyllis vulneraria* appear to be largely outcrossing. Indeed, high levels of genetic diversity seem to be maintained within populations and among individuals of *Anthyllis vulneraria*, which are in line with values reviewed by Nybom (2004). In *Arabis alpina*, we found high F_{IS} values indicative of low rates of outcrossing (Buehler et al., 2012) and generally lower genetic diversity than in *Anthyllis vulneraria*. Of 13 alpine plant species studied by Manel et al. (2012) across the entire European Alps, *Arabis alpina* was in fact the least genetically diverse. Genetic diversity is a key determinant of the potential for local adaptation (Geber and Eckhart, 2005, Blanquart et al., 2013, Cheplick, 2015) and a possible explanation for the absence of evidence for local adaptation in this species is the high level of inbreeding.

When individuals of *Antyllis vulneraria* were transplanted away from their home site, their fitness decreased on average, and more strongly so at larger geographic distance from the home site. Although one would intuitively assume greater divergence in environmental conditions with increasing geographic separation between sites, this is not necessarily given. Indeed, transplantations across range limits can cause much stronger fitness reduction than those across habitat types within the native range, even if the geographical distance of the latter is much greater than that of across-range transplantations (Geber and Eckhart, 2005). This suggests that geographic distance alone is not necessarily a surrogate for ecological divergence. Our results, however, suggest that within the native range of *Anthylllis vulneraria*, geographic distance can substitute for ecological distance and that regional and local environmental variation

sum up. It would be interesting to use a multispecies approach to test the hypothesis whether this is always true within a species' native range. As we have not specifically set out to test the effect of certain environmental variables, it is difficult to pinpoint selective agents responsible for local adaptation in *Anthyllis vulneraria*. However, when comparing the two regions used in this study, Zermatt has substantially warmer and dryer summers than Davos and less snowfall during winters (MeteoSwiss). If we presume that within-region small-scale variation, such as variation in exposition, elevation and inclination are similar in both regions, then the additional regional variation should cause additional differentiation between populations from different regions. Therefore it is conceivable that climatic divergence between the two regions in part explains the fitness variation observed for cross-regional transplants of *Anthyllis vulneraria*.

The patterns of local adaptation in *Anthyllis vulneraria* were most visible in reproductive biomass and flowering propensity. Reproduction and flowering in alpine plants are commonly associated with vernalisation (Wang et al 2009) and photoperiod (Keller and Körner 2003). However, it is unlikely that the pattern of local adaptation in flowering propensity and reproductive biomass found here is caused by adaptive differentiation in vernalisation requirements or photoperiodic control. All populations used here need only little vernalisation: in several independent experiments, the same populations were able to flower in the botanical gardens of our institute at 300 m a.s.l., where winters are much warmer and shorter compared to any of the transplantations sites. Moreover, all populations are situated at roughly equal latitude, and so differentiation according to photoperiod is unlikely. Due to the higher vegetative biomass in *near allopatric* population by habitat combinations (dlsm: *sym-near.allo* = 0.2, p=0.032), we can also rule out reduced growth as the sole cause for lower flowering propensity or the inability to reach reproductive maturity of allopatric transplantations (Angert and Schemske 2005).

Even though we must be cautious when interpreting the current results for *Arabis alpina* due to the low final sample size and short observation period, our reciprocal transplant experiment represents an important direct test for local adaptation in this emerging alpine model organism of the European Alps. Evidence for local adaptation in *Arabis alpina* of the central European Alps until now was based on the detection of outlier loci (Buehler et al., 2012) or association studies of marker loci with environmental variables (Manel et al., 2012), which sometimes rest on statistical assumptions or environmental and molecular data of coarse resolution. In the current experiment, we found only little evidence for the hypothesis that populations of *Arabis alpina* within the Swiss Alps are locally adapted. Along with the high level of inbreeding, a possible explanation for the lack of local adaptation in this species is that the ecologically relevant factors for *Arabis alpin*a are different than those for *Anthyllis vulneraria*, and that the spatial distribution of variation in these factors is not structured along geographic distance. Torang et al. (2015) have conducted a reciprocal transplant experiment with *Arabis alpina* across wide latitudinal gradients (between Spain and Sweden; 3'000 km) and found local adaptation in flowering phenology and other traits related to temperature, water availability, and growing season length. However, they did not find significant fitness

differences between populations of the same region (within 690km circumference). In the present study we use a maximum transplantation distance of 180 km, and therefore both transplantation studies carried out to date with *Arabis alpina* come to the conclusion that local adaptation does not occur at distances smaller than a few hundred kilometers.

Conclusions

We performed reciprocal transplantation experiments to test for local adaptation at two spatial scales using two unrelated but common alpine species. In *Anthyllis vulneraria*, flowering propensity and reproductive biomass decreased with increasing geographical distance to sites of origin, indicating local adaptation in this species. The results provide evidence for the role of natural selection in shaping phenotypes in populations of the European Alps and suggest that environmental differences between sites increase on average with geographic distance. In the highly selfing *Arabis alpina*, no evidence for local adaptation was found across regions, and only little evidence for local adaptation within regions. Whether inbreeding eliminates the potential for local adaptation in *Arabis alpina* at spatial scales smaller than a few hundred kilometres requires further tests.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary Data

Table S1: Pairwise population matrix of Fst values for *Anthyllis vulneraria*. Fst values are shown below the diagonal and probability, P(rand >= data) based on 999 permutations is shown above diagonal.

	Schiahorn	Monstein	Casanna	Findelgletscher	Findelwald	Stafelalp
Schiahorn	0.000	0.001	0.001	0.001	0.001	0.001
Monstein	0.084	0.000	0.037	0.001	0.001	0.001
Casanna	0.051	0.014	0.000	0.001	0.001	0.001
Findelgletscher	0.127	0.096	0.078	0.000	0.014	0.001
Findelwald	0.095	0.064	0.038	0.020	0.000	0.450
Stafelalp	0.098	0.085	0.039	0.052	0.000	0.000

Table S2: Pairwise population matrix of Fst values for *Arabis alpina*. Fst values are shown below the diagonal and probability, P(rand >= data) based on 999 permutations is shown above diagonal.

	Schiahorn	Casanna	Weissfluhjoch	Blauherd	Findelgletscher	Trockener Steg
Schiahorn	0.000	0.001	0.048	0.001	0.001	0.001
Casanna	0.125	0.000	0.001	0.001	0.001	0.001
Weissfluhjoch	0.048	0.127	0.000	0.001	0.001	0.001
Blauherd	0.165	0.163	0.155	0.000	0.003	0.001
Findelgletscher	0.282	0.210	0.266	0.101	0.000	0.001
Trockener Steg	0.181	0.171	0.155	0.177	0.257	0.000

Chapter 8

Novel microsatellite markers for the high-alpine *Geum reptans* L. (Rosaceae)

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Novel microsatellite markers for the high-alpine *Geum reptans* L. (Rosaceae)

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Abstract

• Premise of the study: *Geum reptans* L. reproduces by outcrossing or the formation of stolons. Sexual and clonal reproduction are not exclusive strategies and occur mostly simultaneously. We developed novel microsatellite primers for this species. The microsatellites will be used in a study about local adaptation, phenotypic plasticity and random molecular divergence of alpine plants.

• Methods and Results: Initially, the forward primers had an M13 tail, and the allelic signals of each locus were amplified using a single fluorescent labeled M13 forward sequence. In the running phase, a multiplex PCR assay was developed using different fluorophore-labeled forward primers. Two to eleven alleles were found per locus, depending on the studied population.

• Conclusions: Identical multi-locus genotypes (i.e. clonal offspring) were not found as spacing between individuals in our sampling was at minimum four meters. F_{st}-Q_{st} analysis will be applied to detect selection processes in populations of *Geum reptans* across the Alps.

Keywords: Alpine scree; clonal reproduction; ECO500 size marker; multiplex PCR

Introduction

It is generally assumed that alpine plants are locally adapted due to strong selection in habitats characterized by severe climatic conditions and high environmental heterogeneity (Körner, 2003). However, this assumption has rarely been tested.

Our research uses reciprocal transplantation experiments (RTE) and sibling analyses to estimate the degree of phenotypic plasticity versus the degree of local adaptation in populations of several alpine plant species (Kawecki & Ebert, 2004; Via, 1984). As each transplantation site of an RTE is also a common garden, phenotypic differentiation of plant functional traits can be compared to neutral genetic differentiation based on microsatellite data (i.e. F_{st}-Q_{st} analysis). This allows to infer strength and direction of past selection on the measured traits and therefore to test for local adaptation (Scheepens et al. 2013; Spitze, 1993).

One of our focal alpine plants is *Geum reptans* L. (Rosidae). Cross-amplification of microsatellite primers developed for the lowland rosid *Geum urbanum* has been reported in seeds of *Geum reptans* (Arens et al. 2004). However, trans-species amplification was not successful in our lab although we used the PCR protocol given by Arens et al. (2004). Hence, we decided to find new polymorphic microsatellite loci in *Geum reptans* in order to analyze neutral genetic differentiation of our study populations.

Methods and results

Geum reptans is a diploid, perennial rosette plant usually found in high alpine scree fields and in glacier forelands. The plant uses sexual reproduction to produce viable seeds, and also expands through vegetative aboveground stolons (Fig. 1 and Weppler et al. 2006). Selfing provides no viable seeds in *G. reptans*, probably due to gametophytic self-incompatibility as in other species of the Rosaceae (see Rusterholz et al. 1993).

Fig. 1: Reproducing individual of *Geum reptans* in a glacier forefield. The stolons (red arrows) root at the end, eventually forming a new clonal plant next to its 'mother'. This reproductive mode with sexual flowers and vegetative, above-ground stolons is comparable to strawberries (e.g., *Fragaria vesca*).

We sampled three geographically distinct populations of the Swiss Alps (Davos: 46°44'47.46"N, 9°56'47.70"E; Furka: 46°33'24.28"N, 8°24'45.40"E; Zermatt: 45°59'16.69"N, 7°40'40.73"E). Tissue samples from young leaves of 20 randomly selected individuals were collected from each population in summer 2012. Sampled

individuals were separated by at least four meters to minimize the risk of re-sampling identical clones (Pluess & Stöcklin 2004, p. 2014). Leaf material was stored in Silica in the collection of the University of Basel, section of Population Biology of Plants.

The microsatellite project was outsourced to a professional company for marker development. ECOGENICS GmbH (Schlieren, Zurich, Switzerland) received the Silica dried leaf material of *G. reptans*. The genome screening technique of ECOGENICS has been described previously (Kesselring et al. 2013). The total 26,603 reads had an average length of 178 bp, and 2,222 of these reads contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 309 reads. In order to find allelic polymorphisms a test sample of N = 15 individuals was used. For the screening of loci for polymorphy and PCR functionality, a PCR strategy (see details in Kesselring et al. 2013) that involved M13-tailing at the 5'-end of each forward primer was used (Schuelke 2000). Finally, nine out of twelve loci provided sufficient allelic polymorphisms and robust PCR characteristics (Table 1). In order to facilitate subsequent genotyping, the PCR of the identified loci were combined in three multiplex assays using fluorophore-labeling (Table 1). For each locus, a fraction of the forward primer was labeled with a fluorophore and complemented with non-labeled forward primer and reverse primer to a concentration of 10 µM primer master mix (see footnote in Table 1). Subsequently, 20 individuals from each of the three populations (Davos, Furka, Zermatt) were tested (a total of N = 60 individuals, Table 2). PCR was done in a final volume of 10 µL, and contained 1 µL PCR stock buffer of QIAGEN (Hilden, Germany) with 15 mM $MgCl_2$ and 200 µM dNTP's, 0.3 µL primer master mix of each of the three loci, 0.5 U Hotstar Taq polymerase (QIAGEN, Hilden, Germany), and 2-10 ng DNA. Cycling conditions were: denaturation at 95 °C for 15 min, start PCR at 94 °C 30 sec, 56 °C 90 sec, and 72 °C 60 sec in 35 cycles. Final elongation was set to 72 °C for 30 min. After PCR, samples were mixed with ECO500 size standard (provided by ECOGENICS), and loaded on an ABI3730 sequencer (Applied Biosystems, Carlsbad, California, USA). This size marker is suitable for accurate sizing in the range of 50 – 500 bp. ECO500 was labeled with ‚orange' Dyomics630 dye, and comprised the following basepair fragments: 75, 102, 124, 148, 171, 207, 229, 260, 274, 311, 321, 349, 374, 395, 419, 455, 473, and 497 bp. Allelic assignment of the electropherograms was done with GeneMarker version 1.80 (SoftGenetics LLC, State College, USA). Data were crosschecked for repeatability. Eight of the 60 individuals were re-tested, starting again with DNA extraction of the silica-dried leaves and microsatellite fingerprinting. Hence, 72 fingerprints (i.e., 8 samples · 9 loci) of the first run were opposed to 72 fingerprints of the repetition run. Just one pair-wise comparison differed, which is an error rate of 1.4 %. The error occurred due to inconsistent allelic assignment of an individual at locus 015615. Instead of being heterozygous, the individual was interpreted in the repetition run as homozygous. Two loci showed 'back-ground noise' (Locus 015967 and 013998; Table 1), i.e. we interpreted the constant occurrence of an additional peak as mismatch. Moreover, at locus 013998 an allele of 151 bp was found to occur in a frequency of 5 %. This allele was binned with the common allele of 150 bp because of potential stuttering (see Table 1).

The same was done with the '144' allele (frequency of 8 %; binning with '143') at locus 002235 (Table 1). The polished data set was written in Genepop format (Rousset 2008). Three software packages were used: Genepop on the Web for general index calculations and tests on linkage disequilibrium (http://genepop.curtin.edu.au/), Micro-Checker (van Oosterhout et al. 2004) for tests on potential null-alleles with a prior value of maximum allele length of 250 bp and a 95% confidence limit, and GenAlex 6.2 (Smouth et al. 2008; Beck et al. 2008) for finding identical multi-locus clones.

Two to eleven alleles were found per locus, depending on the studied population (Table 2). Observed (H_o) and expected heterozygosity (H_e) mostly were in good agreement, indicating sexual outcrossing and random mating of alleles. However, three loci showed obvious deviation in H_o vs. H_e (Table 2). The presence of null alleles was suggested by Micro-Checker for locus 002235, 007389 and 011721 in some populations (Table 2). The independent evolution of the microsatellite loci was tested with linkage analysis. There was no linkage disequilibrium among pairs of loci across all populations (all p's > 0.09). In a further step, we searched for identical multi-locus genotypes because of clonal reproduction by stolons. Six of the 60 individuals had to be excluded from the analysis since they had missing values at some loci (000-allele code in Genepop). We did not find identical multi-locus genotypes in the 54 remaining individuals, although the establishment of clonal offspring by stolons of *G. reptans* is common and was estimated to range between 53 % and 74 % (Weppler et al., 2006).

Conclusion

The new microsatellite markers described herein proved to be valuable tools to perform population and landscape genetics studies in the clonal plant *Geum reptans,* for parental analysis or further investigations of its breeding system. Observed and expected heterozygosity were in good agreement, indicating random mating of alleles and sexual outcrossing. Null-alleles might however occur at some loci. Given the absence of identical multi-locus genotypes, we assume that our sampling design was successful in avoiding clonal individuals and indicate that clonal offspring of *G. reptans* establish only right next to their 'mother' plants. In the near future, we will examine microsatellite data of G. reptans to identify neutral genetic differentiation across the Alps. Contrasting molecular differentiation with differentiation in fitness-related phenotypic traits of reciprocally transplanted populations (F_{st}-Q_{st} analysis) should allow detection of selection and local adaptation.

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Table 1: Characteristics of the newly developed microsatellite markers for *Geum reptans*.

Locus [1]	Genbank accession	Primer sequence (5'-3')	Repeat motif	Amplicon length (bp)	Comment
015967 a)	KJ439055	F: ACGGGTCTCTCTTCACTTGG R: TGACCATACTCATTCGCCCC	$(TG)_{13}$	125 - 145	Depending on genotype, mismatch signal between 122 bp and 126 bp.
011721 a)	KJ439056	F: AAAACCCTAGCCTTCGTCGC R: ATGTTAAGTGCAGCGGTTCG	$(TC)_{11}$	92 -121	
013998 a)	KJ439057	F: GAGCCACACTGAAAGCCATC R: GCCACTCTCAGTATCTTCCTCC	$(AC)_{11}$	125 - 150	Depending on genotype, mismatch signal between 124 bp and 125bp. The 151 bp allele was binned with the common allele of 150 bp.
002235 b)	KJ439058	F: TCCGGTCCACCAAAGGATAG R: CTTGCCTTTTCCATGGGCTC	$(CT)_{12}$	143 - 171	The 144 bp allele was binned with the common allele of 143 bp.
003651 b)	KJ439059	F: CCACCTACAGTACGGACGAC R: ACCCCAATTCATTCGACACG	$(GA)_{12}$	125 - 221	
011534 b)	KJ439060	F: CGCCCAAAATCAATCCATCAC R: GTACACCTTTGCTCCCCCTC	$(AG)_{14}$	95 - 189	
015615 c)	KJ439061	F: TTTTGGATTGGACTACATAGACAG R: CAGTACCTGGAATCTGGGGG	$(CA)_{12}$	137 - 160	
013198 c)	KJ439062	F: TGTGATCGATTAACTGCTGACG R: CACTCCCTCCAGCTCAGTTC	$(AG)_{11}$	131 - 182	
07238 [2]	KJ439064	F: ACAAAAATGGCGAGAGCATC R: CTTTGGTACGGCCCATTTCG	$(TGA)_7$	180 - 186	Only eight of the 15 test individuals gave readable amplicons; three alleles with 180 bp, 183 bp and 186 bp were found.
14769 [2]	KJ439065	F: TGTGTGTGTTTTGCCCTAGC R: AAAGTACCCCATCCCAGCTC	$(TC)_{11}$	94	monomorph; 1 allele with 94 bp.
26238 [2]	KJ439066	F: CGTCGCTCTCTCTATCTACCC R: GAGAGTGAGGTTTTCCGGC	$(CCG)_7$	80	monomorph; 1 allele with 80 bp.

[1] Three multiplex PCR assays (a, b, c) were performed. The following fluorescent dyes were used: ATTO532 for F-primer at locus 011721; ATTO565 for F-primers at loci 013998, 011534, and 007389; FAM for F-primers at loci 015967, 002235, and 015615; ATTO550 for F-primers at loci 003651, and 013198. In the multiplex PCR assays, the ratio of fluorophore-labeled F-primer and unlabeled F-primer was between 0.11 and 0.52 (see text). For example, the primer master mix of locus 011721 contained 0.5 µl ATTO532-labeled F-primer, 4.5 µl unlabeled F-primer, 5 µl reverse primer and 40 µl ddH_2O, in a final concentration of 10 µM total F-primer and 10 µM total R-primer. Of this master mix 0.3 µL were pipetted into the PCR tube together with two other primer master mix solutions (see text).

[2] Three of the tested loci were excluded. These loci were either monomorphic in the test sample of N = 15 individuals or could be amplified only in a subset of the 15 test-individuals.

Table 2: Details of three populations from Davos, Furka and Zermatt of *Geum reptans* (each with N = 20 individuals). A = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity.

	Davos			Furka			Zermatt		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
015967	4	0.6	0.57	4	0.75	0.65	2	0.3	0.39
011721	6	0.45	0.66 *	8	0.70	0.79	7	0.45	0.73 *
013998	6	0.60	0.56	3	0.75	0.63	2	0.05	0.14
002235	5	0.50	0.71 *	3	0.35	0.59 *	3	0.10	0.19
003651	11	0.70	0.77	11	0.90	0.83	4	0.65	0.65
011534	8	0.70	0.76	8	0.80	0.86	9	0.85	0.83
015615	9	0.65	0.81	10	0.90	0.86	6	0.60	0.67
013198	9	0.75	0.81	9	0.85	0.85	10	0.90	0.82
007389	9	0.25	0.86 *	5	0.15	0.77 *	4	0.60	0.53

* indicates potential null alleles (see text).

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Chapter 9

New microsatellite markers for Anthyllis vulneraria L. (Fabaceae), analyzed with Spreadex® gel electrophoresis

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New microsatellite markers for *Anthyllis vulneraria* (Fabaceae), analyzed with Spreadex® gel electrophoresis

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Abstract

• Premise of the study: New microsatellite primers were developed for the diploid herb *Anthyllis vulneraria* L. Primers will be used in a study focusing on random genetic variation, local adaptation, and phenotypic plasticity in alpine plants.
• Methods and Results: The new primers were adjusted to separate PCR amplicons (70 bp to 170 bp) on precast Spreadex® gels using horizontal gel electrophoresis. No capillary sequencer was needed.
• Conclusions: Our preliminary results showed that the three studied alpine populations are predominantly outcrossing, but including variable levels of self-fertilization.

Keywords: Alpine plants, Ethidium bromide, Horizontal electrophoresis, Microsatellites

Introduction

Alpine environments are considered to be particularly heterogeneous. Two fundamental survival strategies for heterogeneous environments can be contrasted: local adaptation or specialization vs. phenotypic plasticity, a generalist strategy. A major hypothesis suggests that phenotypic plasticity is favored over local adaptation when the spatial scale of dispersal spans several environmental states (Sultan and Spencer, 2002). Reciprocal transplantation experiments (RTE) are suitable to study both, local adaptation, and the reaction norm of plant phenotypes at different transplantation sites (Kawecky and Ebert 2004). In the near future, we will apply RTE using populations from two spatial scales (representing fine vs. coarse grained environmental variation) of four alpine species including *Anthyllis vulneraria* L. The degree of neutral genetic differentiation will be estimated using microsatellites and will be compared to phenotypic differentiation (e.g., $F_{st} - Q_{st}$ analysis).

Methods and results

In our lab, we used Spreadex® gels and the ORIGINS electrophoresis unit (ELCHROM SCIENTIFIC AG, Cham, Switzerland) for microsatellite analysis. Spreadex® gels resolve PCR amplicons with size differences of 2 bp in an electrophoresis time of 1 to 2 h. Amplicons should not be longer than ca. 170 bp. In nearly all cases, heterozygous character states show a 'third' top band in the gel (i.e. a heteroduplex) because the gels consist of a non-denaturing matrix (see Figure 1, and Armbruster et al., 2005). Homozygous individuals show a single prominent PCR band. For *Anthyllis vulneraria*, we checked the five microsatellite loci AV2, AV3, AV7, AV12, and AV23 and the respective primers described by van Glabeke et al. (2007). These loci promised to be suitable for Spreadex® electrophoresis because the amplicons are between 60 bp and 170 bp. Despite the infraspecific taxonomic uncertainties of *A. vulneraria* (Nanni et al., 2004) the above microsatellites finally proved to be useful for our populations from the Swiss Alps (data not shown). However, to study spatial genetic variation with greater power we needed additional polymorphic microsatellite sequences from the genome of *A. vulneraria*. The development of 10 additional microsatellite primer pairs was outsourced to ECOGENICS GmbH (Schlieren, Zurich; see Matter et al. 2012).

ECOGENICS started with leaf material of *A. vulneraria* from the alpine region of Davos, Switzerland. Size selected fragments from genomic DNA were enriched for simple sequence repeats (SSR) by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The SSR enriched library was analyzed on a Roche 454 platform using the GS FLX titanium reagents (MICROSYNTH AG, Balgach, Switzerland). The total 23,720 reads had an average length of 188 bp. Of these, 574 contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. One prerequisite was that the newly developed amplicons should be in the size range from 70 to 170 bp (see above). Suitable primer design was possible in 120 reads. Subsequently, ten loci (Table 1) provided allelic polymorphisms in 15 individuals (using an 48 capillary ABI3730 sequencer; data not shown). ECOGENICS used M13-tailing at the 5'-end of each forward primer for PCR. Hence, PCR conditions of

ECOGENICS were different from our protocol in the running phase (below). The 10 µL PCR mix of ECOGENICS consisted of 1 µL PCR stock buffer of QIAGEN (Hilden, Germany) with 15 mM $MgCl_2$, 200 µM dNTP's, 0.04 µM forward primer (with M13-tail), 0.16 µM reverse primer, 0.16 µM M13 primer (5'-TGTAAAACGACGGCCAGT-3', labeled with a fluorescent dye for multiplexing), 0.5 U Hotstar Taq polymerase (QIAGEN, Hilden, Germany), and 10 ng DNA. Cycling conditions were: denaturation at 95 °C for 15 min, start PCR at 95 °C 30 sec, 56 °C 45 sec, and 72 °C 45 sec in 30 cycles, continued with 95 °C 30 sec, 53 °C 45 sec, and 72° 45 sec in eight cycles. Termination was set to 72 °C for 30 min.

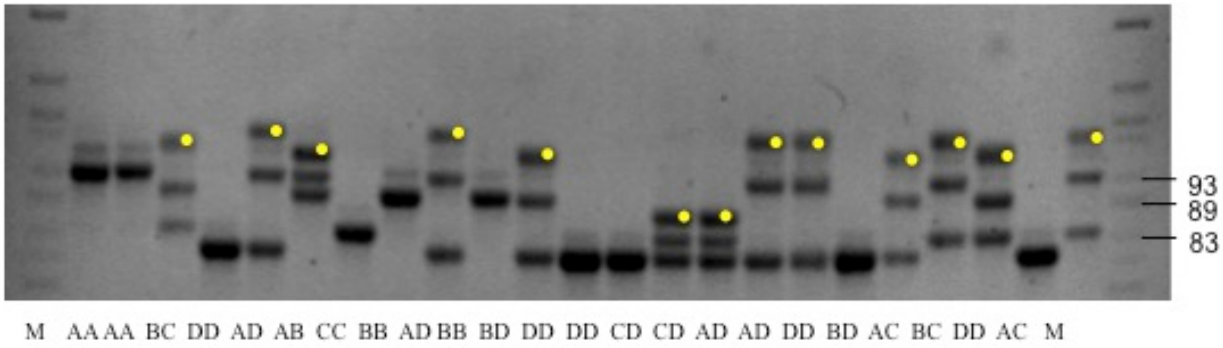

Figure 1. Spreadex® EL 400 gel with electrophoretic resolution of 8 µl to 9 µl of microsatellite amplicons at locus AV-005692. Fingerprints of 23 diploid individuals of *Anthyllis vulneraria* are shown. M = 7 µl of M3 marker from Elchrom Scientific (see bp at right margin). Genotypes are labeled in capitals. Alleles (A,B,C,D) are coded by size (A = 79 bp, B = 83 bp, C = 89 bp, D = 93 bp). Note that heterozygous individuals show a prominent heteroduplex signal (yellow dots).

In the running phase, we checked the ten loci with Spreadex® electrophoresis. Three distinct populations of *A. vulneraria* that were geographically close to Davos, Switzerland, were selected (each with N = 20): Schiahorn (46°48'59.64" N, 9°48'16.80" E), Monstein (46°41'16.92" N, 9°47'15.84" E), and Casanna (46°51'26.88" N, 9°49'37.74" E). Voucher specimens and seeds (sampled by H.K.) are stored in the collection of the University of Basel, section of Population Biology of Plants. DNA was extracted with the DNeasy Plant Mini Kit of QIAGEN (Hilden, Germany). We used self-dissolving illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). 25 pmol forward and reverse primer, ddH_20 and 5 ng of DNA were added to the beads (e.g., Steiner et al. 2012). PCR was run in a MASTERCYCLER GRADIENT (Eppendorf, Hamburg, Germany), with denaturation at 95 °C for 2 min, start PCR at 95 °C 30 sec, locus specific annealing temperature (Table 1) 45 sec, 72 °C 45 sec in 35 cycles. Termination was set to 72 °C for 8 min. Samples were loaded on EL 400 or EL 600 gels (Table 1, Figure 1). M3 ladder from ELCHROM was used as size marker. Finally, gels were stained with ethidium bromide. Nine loci provided PCR amplicons, and their alleles were identical in size (bp) to those reported by ECOGENICS (Table 1). We tested the observed allelic signals for repeatability. Repetition comprised DNA extraction of nine individuals (= 15 % of the 60 individuals; Table 2), PCR and electrophoresis. In the 81 microsatellite lanes on the gels (9 samples · 9

loci), two lanes gave unclear genotype re-assignment (i.e. an error rate of ca. 2.5 %).

Three to twelve alleles were found per locus depending on the population studied (Table 2). Observed and expected heterozygosity (Table 2), linkage equilibrium and Weir&Cockerham F_{is}-values were calculated with 'Genepop (http://genepop.curtin.edu.au/). *P*-values for each locus pair across all populations yielded no significant linkage (all *p*'s > 0.07). The mean F_{is}-values over all loci were positive (Schiahorn = 0.12; Monstein = 0.33, and Casanna = 0.34). Micro-Checker (van Oosterhout et al. 2004) tested for null alleles, with maximum expected allele size set to 200 bp, and a confidence interval of 95 %. No unusual observations were found. Micro-Checker suggested null alleles for AV-021012, AV-021049, AV-021224, and some others (Table 2). However, in the 60 individuals tested just four blank lanes appeared, interestingly all at AV-021049. We believe that 'real' null alleles are therefore only likely for that particular locus. Hence, we suppose that the excess of homozygosity is mostly due to self-fertilization (e.g. three of the 60 specimens were homozygous in all nine loci). Inbreeding is also indicated by the positive F_{is}-values. Autogamy has been reported as the predominant mode of reproduction for French populations of *Anthyllis vulneraria* (see Couderc, 1971), whereas Navarro (2000) found that strong protandry constrained self-fertilization in an Iberian population. The molecular analysis of van Glabeke et al. (2007) of two Belgian populations indicated that they were predominantly outcrossing. As all flowers of an individual plant do not develop synchronously, it is very likely that insects transfer pollen from late flowers to stigmata of early flowers of the same plant (i.e. geitonogamy). Based on our results, we suppose that there is variation in the degree of outcrossing and inbreeding among our populations from the Swiss Alps.

Conclusions

The newly developed microsatellite markers are suitable for horizontal Spreadex® gel electrophoresis with simple ethidium bromide staining and a considerable short electrophoresis time. No sequencer is needed to resolve the allelic patterns. Multiplex of two loci can also be tested, e.g. if the locus-specific amplicons differ in their respective length (e.g, 80 bp to 100 bp *vs*. 110 bp to 130 bp). Central alpine populations seem to be predominantly outcrossing with variable levels of self-fertilization.

Table 1. Characteristics of the newly developed microsatellite markers in *Anthyllis vulneraria*.

Locus	Genbank accession	Primer sequence [1] (5'-3')	Repeat motif [2]	Amplicon length (bp) [3]	T_a (°C)	Spreadex® gel type [4]
AV-000290	KF379737	F: GCAGAGAAGTTATAGTAGCTGTGTG R: CAGCCTGAAAGTATTGGTGGG	$(GA)_{13}$	89 – 123	52	EL 400
AV-002128	KF379738	F: GCATCTAGCCTCGTTTGTTTTATG R: CACTCTTGCGATACGAGAGC	$(TG)_{13}$	77 – 101	52	EL 400
AV-004868	KF379739	F: GTCTGTTTATATGCAATGCGTGC R: CAGCATAGCTGCTTCTGTGAG	$(TG)_{12}(AG)_{12}$	114 – 147	50	EL 600
AV-005692	KF379740	F: TGAAATCAACCCACTAGACAACG R: AACAATCTGGAAACCCTCGC	$(GTT)_7$	77 – 93	52	EL 400
AV-015354	KF379741	F: GACTATGGTGGGTGGGTGG R: TGCGCATACACGAAGAAACC	$(TC)_{11}$	89 – 117	50	EL 400
AV-020270	KF379742	F: ATGAAGGAGGTGGGGCATAG R: TGGGCCATTTGCTTCTATATATGTG	$(CA)_{12}$	136 – 155	52	EL 600
AV-021012	KF379743	F: ACCAGCACCCAAGACCATAG R: TGGAATCGGAGATTGATTCTGG	$(AGT)_8$	82 – 98	50	EL 400
AV-021049	KF379744	F: GGAGCTGCTTTTAGCGAGAG R: GGTCCTCTATGGCAATCCTCC	$(AG)_{17}$	88 – 120	52	EL 400
AV-021224	KF379745	F: TGCATTGTTAAATTGAAGCTAGGTG R: CAGTCGATTCTCCACCCCTC	$(AC)_{18}$	133 – 170	52	EL 600
AV-021803 [5]	KF379746	F: TCTTACTTTCTCACAAGAATGCTATC R: TTTGCTAGTGTTGGACCTGC	$(AC)_{12}$	74 – 104 [5]	---	-----

Note T_a = annealing temperature in the running phase of our project (see text).

[1] Primers used for PCR and subsequent Spreadex® gel electrophoresis with the ORIGINS Elchrom™ electrophoresis chamber. Note that ECOGENICS used F-primers with an M13 tail at the 5'-end, and fluorescent labeled M13 primers in their developmental phase (see text).

[2] Protocol of ECOGENICS, based on genomic DNA sequences analyzed on a Roche 454 GS FLX platform.

[3] In the 60 individuals of Davos (see Table 2), except locus AV-021803 (see footnote 5).

[4] We recommend an electrophoresis time of 1.5 to 2.0 h, a temperature of 55 °C, and 10 V/cm (i.e., 120 V in the ORIGINS Elchrom™ electrophoresis chamber). Our preferred precast Spreadex® gels are the Mini S-2x25 with a loading capacity of 25 samples per gel (loading volume ca. 9 µL per slot). EL 400 and EL 600 differ in the density of the gel matrix. For longer amplicons we used EL 600, for shorter amplicons EL 400.

[5] This locus worked according to the protocol of ECOGENICS (with M13 tailing; see text) but could not be established in the running phase in our lab. Amplicon size is based on 15 individuals checked by ECOGENICS.

Table 2. Details on the three populations of *Anthyllis vulneraria*. A = number of alleles found, H_o = observed heterozygosity, H_e = expected heterozygosity.

	Schiahorn (n = 20)			Monstein (n = 20)			Casanna (n = 20)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
AV-000290	5	0.600	0.601	6	0.600	0.715	8	0.650	0.750
AV-002128	7	0.750	0.695	8	0.650	0.820	8	0.600*	0.802
AV-004868	8	0.850	0.827	8	0.500*	0.729	12	0.600*	0.917
AV-005692	4	0.750	0.675	4	0.500	0.602	6	0.500	0.601
AV-015354	7	0.800	0.770	6	0.550	0.673	8	0.700	0.715
AV-020270	6	0.500*	0.764	5	0.550	0.689	7	0.700	0.764
AV-021012	4	0.700	0.714	4	0.300*	0.670	3	0.150*	0.678
AV-021049	4	0.300*	0.610	8	0.250*	0.720	10	0.200*	0.803
AV-021224	6	0.350*	0.667	6	0.300*	0.635	12	0.400*	0.769

* indicates excess of homozygotes / potential null alleles based on MICRO-CHECKER analysis (see text).

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Chapter 10

General Discussion

General Discussion

Summary

The aim of this thesis was to understand (1) how phenotypic plasticity allows alpine plants to buffer against specific aspects of climate change, (2) if alpine plants harbor the same potential for phenotypic plasticity as lowland species, (3) if patterns of local adaptation are present in alpine plant populations in the Swiss Alps, and (4) to investigate the mutual role of genetic differentiation and phenotypic plasticity in local adaptation in order to infer on a broader scale on the adaptive potential of alpine plants facing climate change. Based on these aims, several questions were formulated in the General Introduction (**Chapter 1**) and were addressed in a number of experiments related in the subsequent chapters (**Chapter 2-9**). I will now attempt to summarize what we have learned from the studies conducted in respect to these questions and emphasize the most novel aspects of our findings.

The first two questions were addressed in a common garden experiment were a large number of congeneric mid- and high-elevation species were grown at two elevations and under different soil water availability (**Chapter 2, and 3**). We specifically examined plastic responses in flowering phenology (**Chapter 2**) and other key functional traits (**Chapter 3**) of alpine species to experimental warming and drought, and asked whether these plastic responses differed in the direction and/or magnitude between congeneric mid- and high-elevation species. Extensive phenotypic plasticity was found for all traits in mid- and-high elevation species and plastic responses seemed to track ongoing climate changes. Flowering phenology was advanced at the lower, warmer site, in accordance with several studies relating earlier flowering phenology in response to warmer temperatures and advanced springtime (Menzel et al., 2006, Cleland et al., 2007). Drought emphasized these responses by further advancing phenological onsets and shortening the duration of phenophases, indicative of an escape strategy limiting the negative effects of reduced soil water availability (Franks, 2011). The major novel finding of this study was the detection of a lower potential for phenotypic plasticity in the flowering phenology of high elevation species in comparison to lower elevation congeners (**Chapter 2**). While a prior study had already related similar findings in deciduous tree seedlings (Vitasse et al., 2013), this study is the first to demonstrate limited plasticity in flowering phenology across a large number of perennial alpine forbs and grasses. We conclude that this result is related to the specific adaptations of high elevation species to a short growing season at high elevation, where selective pressures controlling timing of reproduction become increasingly stronger (**Chapter 2**).

In contrast, while the other examined key functional traits (i.e. specific leaf area: SLA, biomass allocation to roots and reproductive structures; **Chapter 3**) were also very plastic in response to experimental warming and drought, no difference in the magnitude of these plastic responses were detected between mid- and high-elevation species for these particular traits. Aboveground biomass and SLA decreased with elevation and drought for both mid- and high-elevation species. Biomass allocation to roots (RMF) was generally higher in high elevation species relative to lower elevation congeners and drought increased the allocation to reproductive structures (FMF). However, we

found very little evidence for differences in the degree of phenotypic plasticity in key plant traits between mid- and high-elevation species (**Chapter 3**), a result in line with prior studies (Frei et al., 2014b). In contrast to the degree of plasticity of high elevation species in flowering phenology, other key plant traits such as SLA, RMF and FMF were not constrained. These functional traits probably benefit from being very plastic, which allows the adjustment of resource acquisition and allocation and the maintenance of fitness homeostasis across diverse environments. Our results suggest that mid- and high-elevation species respond to warming and drought in a similar way in respect to these key plant traits, and the general capacity of species to respond plastically to environmental changes provides a clear advantage for the persistence and survival of alpine plants (**Chapter 3**). To conclude, these two studies revealed that the degree of functional plasticity in response to changes in environmental conditions is highly trait specific. While, alpine species had a constrained degree of plasticity in flowering phenology relative to lower elevation congeners reflecting their specific adaptation to the alpine environment, this was not a general pattern. In contrast, they benefited from maintaining high plasticity in other traits crucial for fitness homeostasis across diverse habitats, and in the long run, adaptation by means of phenotypic plasticity may allow plants to adapt to the environmental changes via genetic assimilation (Price et al., 2003).

A second common garden study, where source populations of *Anthyllis vulneraria* from two regions in the Swiss Alps were grown together under control or limited soil water availability, was used to compare quantitative trait differentiation in flowering phenology and allocation to reproductive biomass with genetic differentiation at neutral marker loci (**Chapter 4**). In line with previous findings, reduced soil water availability advanced phenophases suggesting an escape strategy. This response was uniform as genetic variation for phenotypic plasticity in response to soil moisture availability was absent across populations. In accordance with prior studies (Frei et al., 2014a), the main finding of this study was the demonstration that the timing of onset and peak flowering has been under past divergent selection ($Q_{ST} > F_{ST}$) among populations of *A. vulneraria* in the Swiss Aps. These results could potentially also indicate local adaptation to currently heterogeneous environmental conditions between population habitats, however, this can only be rigorously demonstrated by reciprocally transplanting populations across their original field sites. As such, **Chapter 4** provides a relevant and fitting transition to our next experiments.

The third question, concerning patterns of local adaptation to present environmental conditions, was investigated in four alpine species differing in life strategies by combining reciprocal field transplantations of populations growing at close or far distance form each other (**Chapter 5, 6, and 7**) with analysis of molecular variation among populations (**Chapter 5, 6, 7, 8, and 9**). For two out of the four studied species (i.e. **Chapter 5**; *Poa alpina* and **Chapter 7**; *Anthyllis vulneraria*), strong evidence was found supporting the hypothesis that divergent selection could lead to local adaptation in the spatiotemporally heterogeneous and fragmented alpine landscape (Kawecki and Ebert, 2004). For *A. vulneraria* the flowering propensity was highest in sympatric transplant combinations,

and decreased with increasing distance between origin and transplant site. In *P. alpina*, results suggested adaption to coarse-grained environmental variability in the reproductive biomass, in line with high regional molecular differentiation. In contrast, the inflorescence height seemed adapted at a finer grain size, suggesting that microhabitat selection was strong. Hence, the spatial scale and the grain size of environmental variability at which transplants were performed were key in identifying patterns of local adaptation, and these patterns were trait-dependent (**Chapter 5**).

For *Arabis alpina*, the evidence for local adaptation is less conclusive as sympatric and far allopatric transplant combinations had equal reproductive biomass, which was however higher relative to near allopatric transplant combinations. Here again, we conclude that environmental divergence does not necessarily increase with geographic distance and may cause complex fitness patterns (**Chapter 7**). Finally, despite high intraspecific phenotypic variation, little evidence was found for local adaptation in *Geum reptans*, a high-alpine clonal plant (**Chapter 6**). We hypothesize that glacier forelands, the typical habitat of this species, are very similar in environmental conditions, and consequently, selective pressures are not divergent or strong enough to cause pronounced adaptive population differentiation. Nevertheless, this last study also revealed high genetic diversity within populations, and low molecular differentiation between populations growing at close proximity, suggesting that the relative high clonality of *G. reptans* does not impede genetic diversity and that gene flow is maintained, at least over short distances.

Methodological limits and perspectives

Common garden and reciprocal transplantation experiments are powerful tools to study genetically based phenotypic differentiation or adaptive phenotypic plasticity. However, like any other scientific method, they are always limited by resources (i.e. time and finances), and by the laboriousness of the task at hand, so that inevitable comprises are struck between the available resources and the optimal experimental design. Furthermore, while one experiment might answer initial questions, they naturally lead to follow-up questions. In this section, I will shortly discuss a few issues concerning experimental limits, and suggest possible guidelines to answer newly raised questions.

While we were determined to include a large number of species in the common garden experiments (**Chapter 2 and 3**), a compromise was made in ordering seeds from seed producers rather than collecting maternal seed families ourselves *in* situ as this would have set us back by an entire growing season. However, this led us to consider phenotypic plasticity at the species level rather than at the narrow-sense genotype level.

Moreover, the drought treatment could have been optimized. Instead of imposing an arbitrary level of drought stress on the plants, precise quantities and frequencies of natural precipitation events could have been followed, to precisely estimate the repercussions of decreasing summer precipitation in the Swiss Alps. This would have involved measuring the quantity and frequency of summer precipitation events and equally re-distributing water amounts

after each precipitation event, to mimic natural periods of drought. Indeed, new experimental designs have been proposed to improve climate change experiments by accounting for the frequency and magnitude of extreme events (Jentsch et al., 2007). In order to increase the accuracy of our predictions regarding the responses of alpine species to summer drought events, follow-up experiments should be conducted using such designs.

Concerning the reciprocal transplantation experiments (**Chapter 5, 6, and 7**), we have come to the conclusion that the experimental design can be optimized. For practical reasons, an inevitable compromise has to be made between sampling many individuals from a small number of populations or a small number of individuals from a large number of populations. We chose the first option as we expected high rates of mortality after transplantations. While we were successful in detecting local adaptation in *P. alpina*, *A. vulneraria* and *A. alpina* despite this strategy, in retrospect, a higher number of populations should have been included in these experiments at the expense of number of individuals. This would have increased statistical power since the population and not the individual is the relevant unit of replication when testing for local adaptation (Blanquart et al., 2013). In the case of *G. reptans*, unfortunate early snowfall limited the number of populations that we could sample. However, we believe that the reason for the lack of evidence for local adaptation is not the small number of populations but rather the low divergence in environmental conditions in glacier forelands where this species grows.

Although most of our reciprocal transplantations were successful in detecting local adaptation in alpine species, they raised the following question: which specific environmental factors are traits adapted to? While some hypotheses were formulated concerning factors varying over coarse or fine environmental grain size (i.e. climate, competition, respectively), our experiments do not allow saying with accuracy. This issue could however be addressed by measuring biotic and abiotic factors at each transplantation site (competition, climate data, soil type etc.), and correlating trait values with environmental data. We initially attempted to do so by installing data loggers at transplant sites, but some were unfortunately lost, and climate data was consequently often obtained from weather stations more or less far from the sites. Thus, our data is not precise and reliable enough to test for correlations, and follow-up experiments are needed to investigate precisely which environmental factors impose divergent selection on plant traits. Such experiments would of course be incredibly challenging, and could be the focus of another doctoral thesis, given the infinite factors that could be considered and the complex nature of ecological interactions.

Conclusions

Using widespread plant species from the Swiss Alps, I explored three main questions in this thesis: (1) does phenotypic plasticity allow to buffer against specific aspects of climate change (i.e. warming and drought)? (2) Do alpine species harbor the same potential for phenotypic plasticity in key functional traits as lower elevation congeners? (3) Are populations of alpine species locally adapted, and what is the relative role of genetic differentiation and adaptive phenotypic plasticity in this process in respect to spatial scale of environmental

variability? When combining the answers to these three questions, this thesis allows inferring on the adaptive potential of alpine species in the context of current climate change.

As such, I hope the studies conducted in this work frame constitute a humble contribution to understanding the repercussions of climate change on alpine plants and their adaptive potential when facing it. While anthropogenic climate change poses an uncontestable threat to mountain biota, our work provides evidence suggesting that the adaptive potential is high in alpine species. All our experiments demonstrated that alpine plants possess a remarkable capacity to respond to changes in environmental conditions by means of phenotypic plasticity, which confers a definitive advantage for survival and persistence in heterogeneous environments (Alpert and Simms, 2002). Moreover, the evidence found for genetic population differentiation and local adaptation in some alpine species, indicates that selective forces are strong and that natural selection is acting on plant populations. Furthermore, we found that within population genetic diversity and gene flow were not necessarily restricted by spatial isolation, small population size or clonality at high elevation (Stöcklin et al., 2009). Hence, the presence of phenotypic plasticity, alongside to within population genetic diversity and the maintenance of genetic breadth among populations, suggests that the potential for adaptive is intact in alpine species. However, the question remains whether natural selection can keep pace with the speed of ongoing changes (Visser, 2008, Shaw and Etterson, 2012).

At this point, I would like to conclude this work on a more personal and optimistic note by saying that, given our results and recent studies that have demonstrated incredibly rapid adaptive evolution in plant populations (Franks et al., 2007, Nevo et al., 2012, Bustos-Segura et al., 2014), a beacon of hope remains to suggest that plant adaptation and persistence may prevail over local extinction in the face of climate change.

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Education

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2015-2016	**Postdoc** at the University of Basel, Switzerland.
2012-2015	**Ph. D**. in Botany and Evolutionary Ecology, University of Basel, Switzerland. Supervisor Prof. Dr. Jürg Stöcklin. Thesis title: "The role of phenotypic plasticity and local adaptation in Alpine plants facing climate change".
2009-2011	**M. Sc.** in Ecology & Evolutionary Biology, University of Lyon 1, France. Supervisor Sara Puijalon (Chargé de Recherche CNRS). Thesis title: "Morpho-anatomical and biomechanical responses of aquatic wetland plants to drought".
2006-2009	**B. Sc.** in Biology, University of Lyon 1, France
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Academic Appointments

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2015-2016	**Postdoctoral Research Associate**, University of Basel, Switzerland.
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Teaching experience

2016	**Co-lecturer**, Contemporary Evolution, Fordham University, USA
2012-2015	**Co-lecturer**, Ecosystem and population processes, University of Basel, Switzerland
2012-2015	**Trilingual guided tours** of the Botanical Gardens, University of Basel, Switzerland

Undergraduate/Graduate Mentoring and Thesis supervision

Ph.D students	Acer VanWallendael, Stephen Johnson, Hansol Lee, Fordham University
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Peer-reviewed Publications

Hamann E, Kesselring H, Armbruster GFJ, Scheepens JF, Stöcklin J (2017) High intraspecific phenotypic variation, but little evidence for local adaptation in *Geum reptans* populations in the Central Swiss Alps. *Alpine Botany*, DOI: 10.1007/s00035-017-0185-y.

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Kesselring H, Armbruster GFJ, **Hamann E**, Stöcklin J (2015) Past selection explains differentiation in flowering phenology of nearby populations of a common alpine plant. *Alpine Botany* (2015) **125**:113-124.

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Kesselring H, **Hamann E**, Stöcklin J, Armbruster GFJ (2013) New Microsatellite Markers for *Anthyllis Vulneraria* (Fabaceae), Analyzed with Spreadex Gel Electrophoresis. *Applications in Plant Sciences* **1**: 1300054.

Hamann E, Puijalon S (2013) Biomechanical responses of aquatic plants to aerial conditions. *Annals of Botany*, **112**: 1869-1878.

Publications in review, submitted or in preparation

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VanWallendael A, **Hamann E**, Franks S. Reciprocal transplantation of Japanese knotweed (*Reynoutria japonica*) reveals phenotypic differentiation, but not local adaptation across a wide latitudinal range. In preparation.

Kesselring H, Scheepens JF, **Hamann E**, Armbruster GFJ, Stöcklin J. Patterns of local adaptation in two common herbs from the central European Alps. In preparation.

Conference contributions

06/2017 **Evolution 2017**, Portland, USA: "Evolutionary responses to repeated drought episodes in *Brassica rapa*".

05/2017 **30th PopBio** conference, Halle, Germany: "Intraspecific phenotypic variation but no local adaptation in *Geum reptans* populations in the Swiss Alps" (poster).

06/2016 **Evolution 2016**, Austin, USA: "Testing for local adaptation in four alpine species using reciprocal transplantation experiments".

09/14 **44th GfÖ** conference, Hildesheim, Germany: "Phenotypic plasticity in functional traits of alpine plants in response to warming and drought".

44th GfÖ conference, Hildesheim, Germany: "Flowering phenology of congeneric lowland and highland species in response to warming and drought" (poster).

05/14 **27th PopBio** conference, Konstanz, Germany: "Shifts in reproductive phenology in response to warming and to drought: a comparison between lowland and alpine plants".

05/13 **26th PopBio** conference, Tartu, Estonia: "Phenotypic plasticity in alpine plants: how do they react to warming and drought and how do they compare with lowland species".

09/11	**Ecophysiology of freshwater organisms Workshop**, Lyon, France: "Biomechanical properties of macrophyte responses to drought".
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Memberships

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International Association of Vegetation Science (IAVS)
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Languages

German	Mother tongue
French	Bilingual
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Experimental and analytical techniques and other qualifications

Common garden, reciprocal transplantation, and greenhouse experiments
Statistical analysis of large datasets with R (linear models, mixed effect models, phenotypic selection analysis, aster models etc.)
Genomic tools (RNA extractions, RNA sequencing, DESeq2 etc.)
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